# STUDIES OF PARATUBERCULOSIS OF RED DEER

JOHN FREDERICK CHARLES NYANGE
B.V.M., DIP. VET. PATH.

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DEDICATION

For my wife Rosemary, my children Mary, Sekela and Charles and my parents.

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# DECLARATION

This thesis was composed by myself. The work described was part of the research programme of a group at the Moredun Research Institute, Edinburgh concerned with investigations of paratuberculosis of deer. Nevertheless the work reported in this thesis was carried out by myself unless otherwise stated and I played a full role in the design and execution of the investigations and interpretation of the results.

John F.C. Nyange

# UNIVERSITY OF EDINBURGH

## ABSTRACT OF THESIS (Regulation 3.5.10)

*Name of Candidate* John Frederick Charles Nyange

*Address* Moredun Research Institute, 408 Gilmerton Road, Edinburgh, EH17 7JH

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Studies of the epidemiology, pathology, pathogenesis and aetiology of paratuberculosis of red deer (Cervus elaphus) are described.

The mesenteric lymph nodes of 167 apparently healthy slaughtered red deer (18-24 m.o. old) from a herd in which several cases of paratuberculosis had occurred were examined by histopathology. An infection rate of 38% was found suggesting a high susceptibility of deer to paratuberculosis. Vaccination of all day-old calves with a standard bovine paratuberculosis vaccine stopped the occurrence of disease in yearlings and reduced significantly the rate (64/167 vs 31/201 $p << 0.001$) and severity (41/64 vs 4/31 $p << 0.001$) of infection as assessed by lymph node pathology of normal slaughter deer.

The pathological lesions of paratuberculosis as observed in naturally and experimentally infected red deer, were inconsistent. Nevertheless, the specific lesions were basically similar to those of the disease in other ruminants, especially sheep and goats, where in severe cases, focal necrosis, mineralization and reactive fibrosis accompanied the granulomatous lesions.

The clinical and pathological findings of avian tuberculosis in red deer, in a single natural case and in experimentally infected animals, were similar to those of paratuberculosis but they were more severe and widely disseminated into organs other than the intestines and related lymph nodes.

The pathogeneses of paratuberculosis and avian tuberculosis in very young deer and very young sheep were shown to be similar by experimental infection. However the two infections progressed more rapidly and severely in deer than in sheep, with avian tuberculosis being more acute and severe than paratuberculosis.

Fifty-four isolates of M. paratuberculosis from red deer were shown by cultural characterization to be similar to one another and to isolates of the organisms from a cow and a goat. Analysis of 14 of the isolates by SDS-PAGE and Western blotting did not distinguish them from one another or the bovine and caprine isolates. Despite many similarities in their polypeptide patterns, they were shown to differ from an atypical isolate of mycobacterium (M928), a commercial protoplasmic antigen (PPA-3) and M. avium isolates. Further analysis of six of the isolates by genomic DNA RE digestion with BamHI, PstI and EcoRI and hybridization with $^{32}$P-PCR279 (a DNA probe specific for M. ptbc), showed them to be homologous to both the bovine and caprine isolates when using BamHI and PstI, and only to the caprine isolate when using EcoRI.

In conclusion, paratuberculosis of deer is basically similar to that of other ruminants, especially sheep and goats. However the short course and severity of disease in young animals is novel, contrasting with cattle, sheep and goats in which older animals become clinically affected.

# ABBREVIATIONS

| | |
|---|---|
| AFB | Acid-fast bacillus |
| AGID | Agar gel immunodiffusion |
| AP | Alkaline phosphatase |
| APDS | Avian tuberculosis deer serum |
| APS | Ammonium per sulphate |
| B663 | Rimino phenazine or G303220 |
| bp | Base pair |
| BSA | Bovine serum albumin |
| BTB | Blot tank buffer |
| BWB | Blot wash buffer |
| $^{o}C$ | Degree Celsius |
| CCA | Conglutinating complement absorption |
| CF | Complement fixation |
| Ci | Curie |
| CIE | Counter-immunoelectrophoresis |
| $cm^2$ | Square centimetre |
| CMI | Cell-mediated immunity |
| CPC | Cetyl pyridinium chloride |
| CVL | Central Veterinary Laboratory |
| DAB | Diaminobenzidine |
| DIA | Dot immunoblotting assay |
| DL-DTT | Dithiothreitol or Cleland's reagent |
| DNA | Deoxyribonucleic acid |
| DTH | Delayed-type hypersensitivity |
| DW | Distilled water |
| ECB | ELISA coating buffer |
| EDTA | Ethylenediaminetetraceticacid |
| ELISA | Enzyme-linked immunosorbent assay |
| ESR | Erythrocyte sedimentation rate |
| EWB | ELISA wash buffer |
| FA | Fluorescent antibody |
| FT | Ferritin |
| G | Gauge |
| xg | Relative gravity |
| GLC | Gas-liquid chromatography |
| HA | Haemagglutination |
| Hb | Haemoglobin |
| H&E | Haematoxylin and eosin |
| HL | Haemolysis |
| HPC | Hexadecyl pyridinium chloride |
| HRP | Horseradish peroxidase |
| i/d | Intradermal |
| IgG | Immunoglobulin G |
| i/m | Intramuscular |
| i/v | Intravenous |
| kb | Kilobase |
| kDa | Kilodalton |
| LF | Lactoferrin |
| LMI | Leucocyte migration inhibition |
| LT | Lymphocyte transformation |
| mA | Milliampere |
| MAFF | Ministry of Agriculture, Fisheries and Food |
| MAIS | Mycobacterium-avium-intracellulare-scofulaceum |
| MCV | Mean corpuscular volume |
| M. ptbc | Mycobacterium paratuberculosis |
| m.o. | Month |
| MRI | Moredun Research Institute |
| MW | Molecular weight |

| | |
|---|---|
| OD | Optical density |
| p | Probability |
| PAGE | Polyacrylamide gel electrophoresis |
| PBS | Phosphate buffered saline |
| PCR | Polymerase chain reaction |
| PCV | Packed cell volume |
| PNPP | p-Nitrophenyl phosphate |
| PPA-3 | Protoplasmic antigen-3 |
| PPD | Purified protein derivative |
| PPDS | Paratuberculosis-positive deer serum |
| PPSS | Paratuberculosis-positive sheep serum |
| p.s.i. | Pounds per square inch |
| RBC | Red blood cell or erythrocyte |
| RE | Restriction endonuclease |
| rev/min | Revolutions per minute |
| RFLP | Restriction fragment length polymorphism |
| s/c | Subcutaneous |
| SDS | Sodium dodecyl sulphate |
| TBE | Tris-borate-EDTA |
| TE | Tris-EDTA |
| TEMED | N',N',N'N-Tetramethylethylenediamine |
| TEN | Tris-EDTA-sodium chloride |
| TF | Transferrin |
| TLC | Thin-layer chromatography |
| u | Unit |
| UV | Ultraviolet |
| V | Volt |
| VIC | Veterinary Investigation Centre |
| VUC | Viable unit count |
| v/v | Volume per volume |
| WBC | White blood cell or leucocyte |
| w/v | Weight per volume |
| yr | Year |
| ZN | Ziehl-Neelsen |

CONCENTRATION

| | |
|---|---|
| M | Molar |
| mM | Millimolar |
| μM | Micromolar |

LENGTH

| | |
|---|---|
| cm | Centimetre |
| mm | Millimetre |
| μm | Micrometre |
| nm | Nanometre |

TIME

| | |
|---|---|
| h | Hour |
| min | Minute |
| sec | Second |

VOLUME

| | |
|---|---|
| l | Litre |
| ml | Millilitre |
| µl | Microlitre |

WEIGHT

| | |
|---|---|
| g | Gram |
| mg | Milligram |
| µg | Microgram |

MISCELLANEOUS

| | |
|---|---|
| e.g. | For example |
| pH | Reciprocal log 10 hydrogen ion concentration |
| vs | Versus |

SYMBOLS

| | |
|---|---|
| α | Alpha |
| β | Beta |
| = | Equal to |
| < | Less than |
| > | Greater than |
| " | Inch |
| % | Percentage |

# INTRODUCTION

Free-living mammals are usually regarded as being relatively resistant to mycobacteria (Hillermark, 1966) and infections are seldom seen (Rankin and McDiarmid, 1968). It is however interesting to note that captive and free-living cervids have frequently been mentioned in the literature in connection with infections by typical and atypical mycobacteria (Bourgeois, 1940, 1944; Witte, 1940; Hopkinson and McDiarmid, 1964; Hime, Keymer, Boughton and Birn, 1971; Jørgensen and Clausen, 1976; Matthews, McDiarmid and Collins, 1981; Gumbrell, 1987; Stuart, Mânser and McIntosh, 1988). The zoonotic implications of tuberculosis have always drawn much attention to the disease in routine inspection of both domestic and wild animals, but little attention is ever paid to the other mycobacterioses, particularly in wild animals.

Paratuberculosis (synonym Johne's disease) is recognized as a widespread disease problem among the domestic ruminants (Blood, Radostits and Henderson, 1983), and a considerable amount of knowledge about the disease in cattle, sheep and goats is available as indicated in the extensive reviews by Julian (1975) and Chiodini, Van Kruiningen and Merkal (1984a) but little is known about the disease in deer and other wild animals.

Paratuberculosis is a specific, infectious, chronic enteritis of domestic and wild ruminants (Williams, Spraker and Schoonveld, 1979). The disease is caused by the bacterium Mycobacterium paratuberculosis (M. ptbc), formerly synonymously known as Mycobacterium johnei (Chiodini et al., 1984a). Paratuberculosis is characterized by progressive emaciation in all species affected, and in cattle by

chronic diarrhoea and thickening and corrugation of the wall of the intestine (Blood *et al.*, 1983). The characteristics of paratuberculosis in deer are assumed to be comparable to those of the disease in cattle, sheep and goats, but recent observations in the United Kingdom (McKelvey, 1987) have indicated differences between deer and the other ruminants in their reaction to *M. ptbc* infection. Considerable variation has been noted in the epidemiology and pathology of the disease in deer, and little is known about the characteristics of the mycobacterial isolates responsible for paratuberculosis in deer.

Paratuberculosis was originally described in cattle in Dresden, Germany by Johne and Frothingham (1895) who considered the disease to be an intestinal form of tuberculosis until Bang (1906) differentiated the disorder from tuberculosis. The first published report of paratuberculosis in deer was from the United Kingdom (McFadyean, 1907). In his report, McFadyean suggested that other ruminants like sheep may not be immune to the disease; and ever since paratuberculosis has been reported to occur naturally in domestic and wild ruminants, and under certain circumstances to occur also in monogastric animals such as horses (Rankin, 1956) and swine (Larsen, Moon and Merkal, 1971). Paratuberculosis has a world-wide distribution (Chiodini *et al.*, 1984a) and blame for its introduction is often given to recent importation of infected stock (Sigurdsson, 1956; Nyange, Mbise, Otaru, Mbasha and Karisian, 1985). The disease is economically important despite lack of accurate estimates of its overall impact (Merkal, 1984). Recent observations of farmed deer in the United Kingdom (McKelvey, 1987) and New Zealand (Van Reenen, 1988) suggest that paratuberculosis is potentially an important problem in deer farming.

The major barrier to progress in the battle against paratuberculosis is the problem of its diagnosis. The disease still remains troublesome in many countries due to the lack of a single, suitable, accurate diagnostic test to detect sub-clinical infections (Merkal, 1973). Consequently the infection has managed to establish itself in herds long before its detection. As deer are known to have a wide distribution and in some instances to share their environment with domestic stock (Libke and Walton, 1975; Chiodini and Van Kruiningen, 1983a), and with the increasing interest in commercial deer farming in the United Kingdom, other European countries and New Zealand (Hamilton, 1986), it is prudent that paratuberculosis is prevented from getting established in deer. In view of the variation in the reaction of deer to M. ptbc infection and the difficulties involved in the diagnosis and control of the disease, the threat of paratuberculosis in deer farming can only be prevented through a better understanding of the disease in the deer.

In view of the limited knowledge about paratuberculosis of deer, the work described in this thesis was therefore aimed at providing background information on its aetiology, pathology, pathogenesis and epidemiology in red deer.

In brief the objectives were to study:

(a) the prevalence of infection with M. ptbc in an affected herd of deer and assess the potential value of vaccination in fighting infection within the herd.

(b) the gross and microscopic pathology of the disease in deer.

(c) the pathogenesis of paratuberculosis in experimentally infected deer.

(d) growth requirements and cultural characteristics of isolates of M. ptbc from deer.

(e) molecular biological characteristics of isolates of M. ptbc from deer.

# CHAPTER 1

## Review of the Literature

### 1.1 Aetiology

Mycobacterium paratuberculosis, the causative organism of paratuberculosis is a small, non-motile, non-spore forming, facultatively aerobic, Gram-positive, acid-fast bacillus (AFB) belonging to the order Actinomycetales, family Mycobacteriaceae and genus Mycobacterium (Runyon, Wayne and Kubica, 1974). The bacterium was first grown in the laboratory by Twort and Ingram (1912). M. ptbc is a rod shaped bacillus measuring 1-2 µm in length and 0.5 µm broad (Smith, 1969) and has a thick, impervious, triple-layered cell wall which has a high lipid content (Bendixen, Bloch and Jørgensen, 1981; Kubo, Moriwaki and Watase, 1983). The lipid cell wall is responsible for the acid-fastness of the organism. However non-acid-fast forms of the bacillus have been reported to occur (Hallman and Witter, 1933). These forms possibly account for the occasional failure to demonstrate organisms microscopically in severely affected tissues. M. ptbc often occur in clumps entangled with one another by a network of intercellular filaments (Merkal, Rhoades, Gallagher and Richie, 1973), and possibly by the high hydrophobicity of their lipid cell wall (De Lisle, 1979a).

#### 1.1.1 Growth requirements and characteristics

M. ptbc is a slow growing organism dependent on mycobactin as an exogenous growth factor for its primary growth in artificial media (Francis, Madinaveitia, Macturk and Snow, 1949; Watt, 1954). This growth factor belongs to a group of closely related cell-associated

iron-binding compounds which are produced under iron-limiting conditions by all mycobacteria but not M. ptbc (Barclay, Ewing and Ratledge, 1985). Mycobactin-dependence may be evaded by an addition of high iron concentration in artificial media (Morrison, 1965; Merkal and Curran, 1974). Some strains of M. ptbc have been reported to lose their mycobactin-dependence and become capable of producing their own mycobactin (Merkal, McCullough and Takayama, 1981). The discovery of other mycobactin-dependent mycobacteria such as certain strains of M. avium (Matthews, McDiarmid, Collins and Brown, 1977), the wood-pigeon bacillus (Jørgensen and Clausen, 1976; Matthews and McDiarmid, 1979; Thorel and Desmettre, 1982) and the Mycobacterium spp isolated from patients with Crohn's disease (Chiodini, Van Kruiningen, Merkal, Thayer and Coutu, 1984b) indicate that mycobactin-dependence is not confined to M. ptbc. The Johne's bacillus requires about 8-12 weeks or more of incubation for its growth in artificial media (Merkal, 1970). A chromogenic sheep strain is known to require an exceptionally long period of incubation of up to nine months for its primary in vitro growth (Gilmour and Angus, 1983). As suggested for M. leprae (Ridley, 1978), the thick impervious cell wall of M. ptbc is undoubtedly a barrier for assimilation of essential nutrients. Consequently this, among other factors, contributes to its slow growth. Further improvements in culture techniques employed for the isolation of M. ptbc have markedly improved growth and recovery rates of the organism. The use in the culture media of the homologous mycobactin J extracted from strains of M. ptbc (Merkal and McCullough, 1982), instead of mycobactin P from M. phlei (Francis, Macturk, Madinaveitia and Snow, 1953), has been shown to increase the growth rate of the Johne's

bacillus to an extent that visible colonies may develop in 4-8 weeks with the incubation time reduced by 2-6 weeks. Furthermore exceptionally fastidious strains failing to grow in media with mycobactin P have been isolated readily in media with mycobactin J (Merkal, 1984).

Cultivation of the Johne's bacillus *in vitro* often requires special treatment. As faeces and other materials presented for isolation of the organism are frequently contaminated by other bacteria and fungi, decontamination with chemicals such as 0.3% benzalkonium chloride or 10% oxalic acid (Merkal and Thurston, 1968; Merkal and Richards, 1972) or more recently 0.75% hexadecyl pyridinium chloride (HPC) (Whipple and Merkal, 1983), and addition of other antibacterial and antimycotic agents before cultivation is often necessary. HPC is becoming more popular than the other decontaminants and has been shown to increase the recovery rate of *M. ptbc* (Merkal, 1984). However the problem of decontaminants is that they are bacteristatic and may even be bactericidal for *M. ptbc* (Chiodini *et al.*, 1984a). They also have no effect on fungal spores and certain bacterial species and strains (Merkal and Thurston, 1968); and even with the use of antimycotic agents (Merkal and Richards, 1972) cultures are often lost by contamination.

Many media are used for the isolation of *M. ptbc*, but the most commonly used are Herrold's egg yolk and serum agar media containing at least 2 mg/l mycobactin (Merkal and McCullough, 1982). The Modified Dubos medium (Smith, 1953) improved by Brotherston, Gilmour and Samuel (1961) is an example of the serum agar media.

The colonial morphology of M. ptbc depends on the culture medium used. After 4-6 weeks of incubation at 37-39°C on modified Dubos medium, M. ptbc forms small, firm, raised, rough, dull-white colonies with irregular margins. By 12 weeks, the colonies may attain a size of up to 2-5 mm in diameter especially if there are few competing colonies. Whereas on mycobactin-Herrold's egg yolk media, Merkal (1970) and Merkal and Curran (1974) described small (1 mm diameter), colourless, smooth, moist, translucent and hemispherical colonies. As incubation continues, the colonies change with age and they become more opaque, larger (4-5 mm diameter) and their morphology changes from hemispherical to mammillate.

### 1.1.2 Biochemical characteristics

Biochemical reactions may be used to distinguish M. ptbc from the other mycobactin-dependent mycobacteria. Strains of M. ptbc form a thermostable catalase, do not reduce nitrates and do not produce niacin. Aryl sulphatase, beta-glucosidase and urease tests are negative. Tween hydrolysis (10 days), while negative for M. avium, is positive for M. ptbc and the wood-pigeon mycobacterium. However strain variation in biochemical reactions does occur.

### 1.1.3 Pathogenicity for laboratory animals

Pathogenicity for laboratory animals is another aid for distinguishing M. ptbc from M. avium and the wood-pigeon bacillus. Whereas M. ptbc is non-pathogenic for chickens, guinea pigs and rabbits within 8-12 weeks of experimental infection, M. avium and the

wood-pigeon bacillus are pathogenic for chickens (Jørgensen and Clausen, 1976; Collins, Matthews, McDiarmid and Brown, 1983) and rabbits (Engbaek and Jespersen, 1966; Jørgensen and Clausen, 1976).

1.1.4 Strain variation

Little work has been done on comparison of strains of M. ptbc and their relationship to other species of mycobacteria (De Lisle, 1979a). Strains producing yellow-orange pigment occasionally have been recovered from sheep (Taylor, 1951) and also from cattle (Watt, 1954; Stuart, 1965b). Although unusual strain variation does occur and several variants of the organism from sheep and cattle have been described and characterized by differences in their growth properties and cultural characteristics (Taylor, 1951; Thorel and Valette, 1979), there is no evidence to suggest obvious differences in the pathogenicity for cattle of the different strains of M. ptbc (Hole, 1958).

Apart from cellular morphology, acid-fastness, growth, cultural and biochemical characteristics, and pathogenicity tests, other methods have been employed in attempt to compare strains of M. ptbc and to distinguish the organism from other mycobacteria. These include serotyping, antibiotic sensitivity and biochemical analysis of the various cell components of the organism. Serotyping schemes have largely proved ineffective in distinguishing M. ptbc from other mycobacteria because of the rough morphology of many strains of the organism which causes autoagglutination (Chiodini et al., 1984a). Antibiotic sensitivity is used to differentiate some mycobactin-dependent species, however variation among strains does

occur (Chiodini, 1986). The most promising methods for identification of M. ptbc, comparison of its strains and differentiation from other mycobacteria appear to be the biochemical analysis of the various cell components of the organism. Among the biochemical techniques attempted are chromatographic analysis of fatty acids (Campbell and Naworal, 1969; Coloe, Slattery and Lightfoot, 1983; Craig and Burdett, 1983); thin-layer chromatographic (TLC) analysis of mycobacterial lipids (Brennan, Souhrada, Ullom, McClatchy and Goren, 1978), a method widely used to differentiate members of the Mycobacterium-avium-intracellulare-scrofulaceum (MAIS) group (Brennan, Heifets and Ullom, 1982; Saxegaard, Andersen and Jantzen, 1983). Although Jenkins (1981) considered TLC to show little promise as an aid in identifying M. ptbc because of absence of a specific lipid pattern, Damato, Knisley and Collins (1987) were able to differentiate M. ptbc from other mycobacteria by this method. Gas-liquid chromatography (GLC) (Damato et al., 1987), and GLC in conjunction with growth rate and chromogenicity (Chiodini and Van Kruiningen, 1985) have been reported to be capable of identifying M. ptbc, and distinguishing it from other mycobacteria. More recently, restriction endonuclease (RE) analysis of chromosomal DNA (Collins and De Lisle, 1986; Whipple, Le Febvre, Andrews and Thiermann, 1987) and Southern hybridization (Hurley, Splitter and Welch, 1988; McFadden, Thompson, Hull, Hampson, Stanford and Hermon-Taylor, 1988) have been shown to be potential techniques for genomic comparison and differentiation of slowly growing mycobacteria. From recent studies on the antigenic relationship of the surface glycolipids of a wide range of M. ptbc isolates and the M. avium

complex, Camphausen, Jones and Brennan (1988) have proposed M. ptbc to be an organ- or host- adapted antigen-deficient rough variant of M. avium/M. intracellulare.

### 1.1.5 Inactivation by physical and chemical agents

The survival and viability of M. ptbc is an interesting feature of the organism, significant in the epidemiology of paratuberculosis. The bacillus can survive over extended periods outside the host and can resist a number of disinfectants used commonly in veterinary practice (Lovell, Levi and Francis, 1944; Hole, 1958). The organism has been shown to remain viable for 163 days in river water (Larsen, Merkal and Vardaman, 1956), 270 days in pond water (Jørgensen, 1977) and 11 months in bovine faeces and black soil (Višnevskij, Mamačev, Černyšev and Černyšev, 1940); and to survive freezing at -14$^{o}$C for at least a year (Richards and Thoen, 1977). Faeces are reported to be bacteristatic and urine bactericidal to M. ptbc (Larsen et al., 1956). Despite resistance of the organism to various chemicals, there is evidence to show that the Johne's bacillus is susceptible to certain disinfectants (Vardaman, 1954; Hole, 1958). The organism can be killed within 10 minutes after exposure to any of the following chemical agents: 5% formalin, cresylic disinfectants (1/30 dilution), phenol (1/40 dilution), mercury bichloride (1/1000 dilution) and calcium hypochlorite (1/50 dilution).

## 1.2 Epidemiology

### 1.2.1 Host range

Paratuberculosis is principally a disease of cattle and less often

of sheep and goats (Blood et al., 1983) but other domestic ruminants such as llama (Appleby and Head, 1954; Rankin, 1958b), water buffalo (Katic, 1961; Ganke, Dubaa, Tumbaa and Abugaliev, 1964; Amand, 1974); and a wide range of wild ruminants have also been reported to contract the disease naturally. However few of the reports of paratuberculosis in wild ruminants are from free-ranging animals. The majority of the cases are from farmed and captive animals in parks and zoological gardens (Table 1.0).

Monogastric animals are unnatural hosts of paratuberculosis. However animals such as mules (Eveleth and Gifford, 1943), swine (Runnells, 1955; Jørgensen, Berg and Ringdal, 1962; Jørgensen, 1969; Larsen et al., 1971), horses (Rankin, 1956; Larsen, Moon and Merkal, 1972) and pigmy asses (Van Ulsen, 1970) have been reported to become infected either spontaneously or experimentally, but with few exceptions, without developing the clinical disease. Such asymptomatic animals are carriers of the infection, capable of shedding organisms and thus posing a threat to susceptible ruminants.

Lesions produced by accidental human self inoculation with a killed Johne's disease vaccine have been documented (Bjornsson, Hallgrimsson, Georgsson and Palsson, 1971), but the human analogue of paratuberculosis is the granulomatous ileocolitis produced in Crohn's disease in which an organism closely resembling the Johne's bacillus has been implicated (McFadden, Butcher, Chiodini and Hermon-Taylor, 1987).

Attempts to reproduce paratuberculosis in laboratory animals, as it occurs in ruminants, have been made. Despite replication of the organism within some laboratory animals, the lesions which have

developed after protracted periods have not been consistent with those of the classical disease. Failures to reproduce typical lesions of paratuberculosis experimentally have been experienced with guinea pigs (Twort, 1914; McEwen, 1939; Mohler, 1939; Francis, 1943), rabbits (Mohler, 1939; Francis, 1943; Hirch, 1956; Hole, 1958; Harding, 1959), hamsters (Francis, 1943; Johnson and Pratt, 1944; Hirch, 1956; Hole, 1958; Harding, 1959; Brotherston and Gilmour, 1963; Larsen, Cysewski and Miller, 1975; Larsen and Miller, 1978), cats (Johnson and Pratt, 1944), mice (Lominiski, Cameron and Roberts, 1956; Harding,1959; Chandler, 1961b, 1964; Brotherston and Gilmour, 1963), rats and voles (Hole, 1958), gerbils (Hole, 1958; Larsen, Miller and Kopecky, 1976); and birds such as domestic fowls and pigeons (Mohler, 1939; Larsen and Moon, 1972). A typical strain of M. ptbc inoculated into chick embryos did not replicate (Stavisky and Beck, 1946). However successful infection comparable to that occurring in cattle has been reproduced in rabbits (Twort and Ingram, 1913; Twort, 1914; Rankin, 1958c), mice (Twort, 1914; Francis, 1943), voles (Levi, 1950) and hamsters (Gilmour, Campbell and Brotherston, 1963).

### 1.2.2 Host susceptibility

A number of factors have been found to influence the development of clinical paratuberculosis following infection. Among others, age at first infection is considered to be the most important factor (Hagan, 1938). Young animals particularly newborns are the most susceptible to infection (Doyle, 1953; Rankin, 1959, 1961b), and resistance usually develops with age (Rankin, 1962; Larsen, Merkal and Cutlip, 1975). However adults have also been reported to become infected

without showing clinical evidence of the disease (Rankin, 1961a; De Lisle, Seguin, Samagh, Corner and Duncan, 1980). The nature of this age-dependent resistance is unknown. Bendixen and others (1981) proposed an activation of macrophages resulting in increased bactericidal capacity as a possible mechanism for the resistance. The age susceptibility/resistance of deer to M. ptbc has never been investigated, although the clinical disease has been reported to be predominantly recognized in young animals (1-2 years of age) (Libke and Walton, 1975; Williams, Snyder and Martin, 1983; McKelvey, 1987). This important epidemiological anomaly in the deer deserves investigation. Size of the first and subsequent doses of infection has also been suggested to be a significant factor (Rankin, 1959; Chandler, 1961a). Stress of poor nutrition, parturition, heavy milk production, concurrent infection (e.g. parasitism), introduction to new herds, and transportation and handling have been associated with development of the disease (Julian, 1975; Blood et al., 1983). However the actual influence of stress factors on individual susceptibility to paratuberculosis is obscure. Soil acidity, and calcium (Jansen, 1948) as well as phosphorus deficiency (Fouquet and Delauney, 1960) have also been reported to predispose to the clinical disease in cattle. Intensification, which is associated frequently with high stocking densities, housing and pasture improvement and rotation (Van Reenen, 1988) has been suggested to facilitate environmental contamination.

Familial susceptibility of certain lines of cattle in some herds has been noted and considered to be a significant factor (Hole and Maclay, 1959). The higher incidence of clinical paratuberculosis among certain breeds of cattle has suggested the possibility of breed susceptibility.

In a survey of paratuberculosis in cattle in Great Britain, the incidence of the disease in Channel Island breeds and Shorthorns was higher than in other breeds (Doyle, 1956; Withers, 1959); and in Denmark, Jørgensen (1972) reported Jersey herds to be comparatively more susceptible than the other breeds. In Minnesota and Ontario, the Shorthorn breed was found to have the highest frequency of paratuberculosis (Julian, 1975). An equal frequency of the disease in Holsteins and Guernseys in New York State was reported by Chiodini and co-authors (1984a). In comparison, beef cattle have been observed to have a lower incidence of paratuberculosis than dairy cattle. This is probably because beef cattle generally range over great areas and have less exposure to other cattle's excrement than dairy cattle (Chiodini et al., 1984a). Apart from the reported variation in susceptibility to infection with M. ptbc of different breeds of mice (Chandler, 1961b), this breed variation has not been investigated in species other than cattle. It has been suggested that the apparent familial and breed susceptibility to clinical paratuberculosis may be due to increased exposure rather than increased susceptibility to M. ptbc infection (Blood et al., 1983).

### 1.2.3 Distribution

Paratuberculosis has a world-wide distribution, and it is of major importance in cattle and sheep in temperate climates and some humid tropical areas (Blood et al., 1983). The disease is widespread in cattle in Europe and has been carried to many countries in all continents, except Antarctica, by the export of livestock; and in those

countries where paratuberculosis has never been reported, it may be a matter of lack of diagnostic evidence rather than true absence of the infection.

Paratuberculosis in deer has been reported only in the few countries with farmed deer, which include the United Kingdom (McFadyean, 1907; Buxton, 1987; McKelvey, 1987), Switzerland (Bourgeois, 1940, 1944), the Soviet Union (Hillermark, 1966), the United States and Canada (Vance, 1961; Libke and Walton, 1975; Riemann et al., 1979; Chiodini and Van Kruiningen, 1983a), Sweden (Hillermark, 1966), Denmark (Jørgensen and Jørgensen, 1987) and New Zealand (Gumbrell, 1987).

### 1.2.4 Prevalence

Few surveys on the prevalence of infection with M. ptbc in clinically normal herds have ever been conducted and most of these have been in cattle. Culture surveys of mesenteric lymph nodes of apparently normal abattoir cattle carried out in Great Britain by several investigators between 1947 and 1954 showed an infection rate by M. ptbc varying between 7 and 17% (Taylor, 1949; Rankin, 1954; Smith, 1954b); and a very low prevalence rate of 0.8% was recorded in cattle imported from Ireland where there was little or no Johne's disease (Rankin, 1954). In more recent surveys of paratuberculosis in cattle in the United States, infection rates of 11% in Wisconsin (Arnoldi and Hurley, 1983) and 18% in New England (Chiodini and Van Kruiningen, 1983b) were reported.

In chronically affected herds, clinical cases and deaths due to paratuberculosis represent a small proportion of infected animals. The

annual rate of clinical cases in an affected herd can vary between 1 and 5% , but the infection rate can be greater than 50% (Withers, 1959; Sherman, 1985). The extreme difference between the prevalence of infection and death rates indicates that the majority of the animals infected with M. ptbc become carriers and do not develop the disease.

Knowledge of the prevalence of M. ptbc infection in species other than cattle is sparse. However sheep are considered to be important carriers of the infection for cattle (Blood et al., 1983). A serological investigation of an affected herd of 400 red deer in the United Kingdom (McKelvey, 1987) identified 8.7% seropositive animals.

### 1.2.5 Economic significance

In affected herds, losses due to clinical paratuberculosis may be insignificant, but when added to the hidden costs involved in the protracted periods of poor health, reduced productivity and increased susceptibility to other infections, the disease may cause severe economic embarrassment. In a nationwide survey carried out in Great Britain in 1957-1958, paratuberculosis was found to account for about 0.45% of cattle losses, and to be the most important cause of total depreciation of market value, exceeding mastitis, injuries and accidents, and abnormalities at calving (Ministry of Agriculture, Fisheries and Food, 1957-1958). Judged by a number of criteria, infected animals are less profitable than non-infected herdmates. In the Netherlands, Huitema (1962) found that on affected farms, the average lifespan of dairy cows was a full lactation less than on farms free from the infection. Sub-clinically infected cows appear to be more prone to mastitis and infertility problems (Merkal, Larsen and

Booth, 1975); and their milk production may be as much as 8% lower than non-infected herdmates (Buergelt and Duncan, 1978). It is difficult to ascertain the true financial loss due to paratuberculosis, however in the United States, estimated annual losses exceeding 52 million US dollars in Wisconsin (Arnoldi and Hurley, 1983), and over 54 million US dollars in New England (Chiodini and Van Kruiningen, 1983b) have been reported.

In sheep and goats , estimates of losses ranging between 5 and 15% in affected flocks have been reported (McEwen, 1939; Sigurdsson, 1956; Stamp, 1956). In deer, losses resulting from death and impaired productive capacity have been reported (McKelvey, 1987). In addition, the disease is likely to have a drastic effect on properties wishing to export live deer, embryos and semen to countries demanding freedom from paratuberculosis in the herds of origin (Van Reenen, 1988). Infected herds of deer may also serve as a reservoir of infection for domestic ruminants on shared pastures.

### 1.2.6 Transmission and mode of infection

Paratuberculosis is primarily transmitted by the faeco-oral route (Sherman, 1985). Infected manure contaminating water, pasture and feed is the main source of infection (Larsen, 1972). Newborn animals get infected usually through suckling from teats contaminated with infected faeces (Doyle, 1954) and possibly milk from clinically infected dams (Taylor, Wilks and McQueen, 1981). In experimental infection, the parenteral route of infection was found to be superior to oral dosing in the production of lesions (Chandler, 1961a; Kluge, Merkal, Monlux, Larsen, Kopecky, Ramsey and Lehmann, 1968). The isolation of M. ptbc

from the uterus and foetus in cattle (Pearson and McClelland, 1955; Lawrence, 1956; McQueen and Russell, 1979) suggests that congenital infection does occur; and the recovery of the organism from semen (Lukashaw, Rotov and Rozniatoushaegen, 1962; Larsen and Kopecky, 1970; Larsen, Stalheim, Hughes, Appell, Richards and Himes, 1981), gonads (Tunkl and Aleraj, 1965; Larsen *et al.*, 1981) and accessory glands (Larsen and Kopecky, 1970) of bulls, and survival of the bacillus in semen after commercial processing suggest that breeding by artificial or natural means is also a potential mode of infection. However transuterine infection with subsequent development of the disease *in utero* has never been documented; and experimentally, Merkal, Miller, Hintz and Bryner (1982) found the intrauterine inoculation of contaminated semen to be insignificant in the natural transmission of paratuberculosis in cattle. Chiodini and co-authors (1984a) are of the view that the various extraintestinal modes of infection probably contribute little to overall infection rates.

## 1.3 Pathogenesis

Generally the outcome of mycobacterial infections is depedent on a number of factors among which are the host's age, genetic susceptibility and immune response and characteristics of the infecting strain (Chaparas, 1982). *M. ptbc* is of low pathogenicity for the various species of its natural host range (Chandler, 1961a). This low pathogenicity could be attributed to its slow growth which probably accounts for the long incubation period and chronic nature of the disease.

Although the oral route is the natural mode of infection of paratuberculosis, the primary site of infection is the intestinal tract, with no evidence to date that the tonsils and retropharyngeal lymph nodes become infected first. According to Gilmour (1965) and Gilmour, Nisbet and Brotherston (1965), M. ptbc has a predilection for the intestinal tract. It has been shown experimentally in cattle and sheep (Gilmour et al., 1965) that after ingestion of the organism, it localizes in the intestinal wall where it multiplies in the mucosa before spreading to the regional lymph nodes. The organisms have been shown to enter the intestinal mucosa through the M-cells of the ileal dome-epithelium (Momotani, Whipple, Thiermann and Cheville, 1988). The Johne's bacillus does not produce toxins or virulent factors similar to those of M. tuberculosis (Chiodini et al., 1984a). Once the organism has gained entry into the intestinal wall, it is engulfed by macrophages where it multiplies intracellularly without causing much damage or destruction of the host cell (Sheather, 1932; Hallman and Witter, 1933; Kim, Sanger and Whitenack, 1976; Bendixen, 1978). As the bacilli multiply in the macrophages, they induce a granulomatous reaction in the intestinal mucosa and mesenteric lymph nodes. This ensuing immunological response is thought to be related to a hypersensitivity-type reaction to M. ptbc antigens (Merkal, Richard, Thurston and Ness, 1972). The attempt by macrophages to arrest the organisms and the unimpaired intracellular multiplication of bacilli results in unlimited release of soluble factors which continue to recruit inflammatory cells resulting in distortion of tissues which compromises vascularity and lymphatic drainage.

Lesions in the intestinal wall and the regional lymph nodes have been reported to develop within 1-2 months after infection. However after this phase of intestinal infection, some animals recover, some remain infected for long periods without developing the disease and some become clinical cases sooner or later (Gilmour, Angus and Mitchell, 1978). According to Larsen (1973), these sub-clinical carriers can either be shedders or non-shedders of the organism, and the shedding can either be heavy, light or intermittent. Heavy shedders usually become clinical cases within 1-2 years. However some light shedders can also become clinical cases. Clinical signs of the disease usually appear after establishment of the infection, but the extent and severity of gross lesions in the intestinal wall have been found in many cases not always to correspond to either the extent of the microscopic lesions or to the severity and duration of the clinical signs (Hallman and Witter, 1933). Microscopic lesions have been observed frequently to be much more extensive than would be indicated by gross examination, and the clinical signs to be often more severe than might have been expected fom the extent and degree of the gross lesions. This lack of correlation is particularly common in sheep and goats (Jubb, Kennedy and Palmer, 1985) and it has also been observed in deer (Buxton, 1987; McKelvey, 1987). The discrepancy between the magnitude of the lesions and the degree of the clinical signs has been suggested to be due to immunologic factors e.g. episodes of delayed-type hypersensitivity (DTH) and cytokines (Chiodini et al., 1984a).

In advanced cases, the infection may spread from the intestinal tract and mesenteric lymph nodes, and possibly the tonsils and retropharyngeal lymph nodes, to other sites of the body by the circulating infected macrophages (Payne and Rankin, 1961a, 1961b; Gilmour et al., 1965; Kluge et al., 1968). Buergelt, Hall, McEntee and Duncan (1978) suggested that in organs like the liver, these macrophages may be focally trapped in sinusoids to give rise to microgranulomas.

A number of phenomena have been proposed to contribute to the development of clinical signs of paratuberculosis. Merkal, Kopecky, Larsen and Ness (1970) suggested the release of histamines from mast cells during antigen-antibody reactions, and possibly DTH to contribute to the development of the diarrhoea in cattle. Administration of antihistamines to cows with diarrhoea has resulted in cessation of the diarrhoea, supporting the suggestion. A syndrome of massive plasma protein loss through leakage across the gut wall into the lumen (Patterson, Allen and Lloyd, 1967; Allen, Berrett and Patterson, 1974); and amino acid malabsorption due to space occupying lesions in the intestinal mucosa and lymphatic channels (Patterson and Berrett, 1968, 1969), have been incriminated as major contributory factors to the wasting which occurs during the chronic disease. Merkal and Witzel (1973) have suggested the possibility that the cell-mediated immune (CMI) response may be responsible for the emaciation, anaemia and sometimes febrile response which occur in cases of paratuberculosis. Secondary endocrine lesions, as described in goats infected with M. ptbc, have been incriminated in contributing to the slow growth, poor development and generally poor performance of

infected animals (Rajan, Valsala, Mariamma and Nair, 1980). The secondary endocrine lesions and other atypical degenerative changes in chronic cases of paratuberculosis are probably a manifestation of immune-mediated reactions (Chiodini et al., 1984a) and/or metabolic derangement.

## 1.4 Clinical syndrome

Paratuberculosis manifests itself as a chronic, insidious disease with a protracted incubation period of a few months to several years. In cattle, clinical signs have been observed in animals as young as four months (Smythe and Christie, 1950) and as old as 15 years (Macindoe, 1950); but in most cases, the clinical syndrome is evident at 3-5 years of age. The disease in young stock is known to be rare, except in herds with a high rate of infection coupled with poor husbandry. However in deer, clinical paratuberculosis is reported to be quite uncommon in adults (McKelvey, 1987). Clinical paratuberculosis is the terminal stage of the slow, chronic, sub-clinical infection and may be enhanced by stress factors (Julian, 1975) and other precipitating factors (Smythe, 1935; Van Reenen, 1988); although clinical breakdowns have also been reported to occur in the absence of obvious predisposing or precipitating factors (Jubb et al., 1985).

The typical clinical picture in all species affected is that of chronic wasting, and the outstanding features in cattle are muscle wasting and diarrhoea (Blood et al., 1983). It has been noted that the slight weight loss that may precede the diarrhoea often passes

unnoticed, but the development of the non-responsive, persistent or intermittent diarrhoea is usually dramatic (Sherman, 1985). The diarrhoea is characteristically homogenous and "paint-like" and it is often noticed a few weeks after parturition. Initially it is intermittent, but later it becomes persistent. Other clinical signs include a rough, fading haircoat and dry skin. Inappetance and fever are usually not observed in clinical paratuberculosis. However Merkal and Witzel (1973) reported intermittent bouts of low grade pyrexia. During the last stages of the disease, appetite is lost, the diarrhoea may contain frank blood, and usually there is emaciation and debilitation terminating in recumbency and death.

In sheep (McEwen, 1939; Stamp and Watt, 1954) and in goats (Levi, 1948), clinical para-tuberculosis is comparable to the disease in cattle, but diarrhoea is usually not a prominent feature, and faeces may only be softly pelleted or pasty. In these two species, chronic wasting and generalized unthriftness may be the only presenting clinical features.

In deer, the early stages of the disease have been noted to be difficult to recognize, but as the disease progresses, affected animals fail to gain weight and their winter coat is not shed as rapidly as normal. Weight loss has been observed to precede diarrhoea although some animals have been noticed to die without exhibiting a terminal diarrhoea. Their faeces may only be soft and unformed (McKelvey, 1987).

In cattle, the clinical course of the disease may be acute lasting only a few weeks, or chronic lasting several months to a year or more.

The chronic course may be interrupted by bouts of clinical improvement. In sheep and goats, the course of clinical paratuberculosis as in cattle is variable, but it may progress more rapidly.

## 1.5 Pathology

The principal pathological lesions of paratuberculosis in ruminants are mainly confined to the intestinal tract and mesenteric lymph nodes.

### 1.5.1 Gross pathological lesions

As described by Jubb and co-authors (1985), typical gross lesions of paratuberculosis are seen in cattle in fatal cases and where the animal is slaughtered in an advanced stage of the disease. The carcase is emaciated, and has gelatinous body-fat depots. Intermandibular oedema and serous effusion into body cavities may also be evident, but the latter is more voluminous in small ruminants than in cattle. The hair coat may appear rough and dull, and the skin dry.

The pathognomonic lesions of paratuberculosis are found in the distal small intestine, ileocaecal valve, caecum, proximal colon and the draining mesenteric lymph nodes (Hallman and Witter, 1933), but in severe cases, the lesions may extend towards the duodenum and the rectum.

The gross intestinal lesions of paratuberculosis may be segmental or diffuse (Hallman and Witter, 1933). The earliest macroscopic intestinal changes of the disease occur in the region of the ileocaecal valve, and the first recognizable change is a fleshy, velvety thickening of the intestinal mucosa (Jubb et al., 1985). This change is most common in goats and sheep. In advanced cases of the disease in

cattle (Jubb et al., 1985), the intestinal wall is thickened, sometimes oedematous, and the mucosal surface has thick, broad and closely packed transverse folds resembling the convolutions of the cerebral cortex. This corrugated appearance cannot be smoothed out when the intestine is stretched, and the intestinal wall may fissure if bent sharply. The mucosa is usually pale and glistening, but sometimes there are varying degrees of hyperaemia especially on the top of the corrugations. The mucosa may exhibit minor cracks giving it the appearance of Morocco leather. In less severe cases, the intestinal mucosa may appear granular or diffusely opaque. In animals infected with the pigmented strains of M. ptbc, the mucosa may have a yellow-orange colour, but this change is confined to sheep infected with the yellow strain of the organism (Gilmour and Angus, 1983), and it has never been reported in goats. According to Smythe (1935), some animals suffering from a more acute form of the disease may show no evidence of thickening or corrugation of the bowel, but haemorrhagic enteritis may be present.

Macroscopic features of the mesenteric lymph nodes are quite variable. The nodes may show little or no change or they may be enlarged, oedematous and pale with little corticomedullary distinction. The lymphatic vessels may also show little change or may be prominently thickened into cords which can be traced from the intestine through the mesentery to the draining lymph nodes (Jubb et al., 1985).

In bovine paratuberculosis, necrosis is rare, and caseation and reactive fibrosis do not normally occur. However Richards and Muhm (1972) isolated M. ptbc from a calcified lymph node obtained from a cow

at routine slaughter. Buergelt and co-workers (1978) and Momotani and Yoshino (1985) reported caseous granulomas of mesenteric lymph nodes from cattle infected with M. ptbc.

Lesions outside the intestine and mesenteric lymph nodes are rare, but they may occur in advanced cases of paratuberculosis. When they occur, they are found most commonly in the liver (Mathews, 1930; Buergelt et al., 1978). However the lesions are usually not readily visible by gross examination.

In sheep (Stamp and Watt, 1954) and in goats (Levi, 1948; Harding, 1957; Majeed, 1972; Fodstad and Gunnarsson, 1979) as in cattle, the characteristic lesions of paratuberculosis are also found in the lower intestinal tract and mesenteric lymph nodes, but obvious thickening of the intestinal wall is quite uncommon, except in cases of infection with the chromogenic strain of M. ptbc in sheep. The intestine is usually very friable and readily torn apart during separation from its mesenteric attachment. The intestinal serosa may be slightly granular and diffusely opaque. In the opened intestine, the mucosa has a tendency to crack especially when bent over the finger. Thickening of the mucosa is usually slight and difficult to appreciate. As in cattle, lymphatic lesions are variable and they may be grossly inapparent. The mesenteric lymph nodes may be markedly enlarged, and in some cases with obvious cortical widening. Oedema of the nodes and attaching mesentery does occur. The afferent lymphatics may be corded and knotted and nodules may also be found subcapsularly in the mesenteric lymph nodes. Focal necrosis, caseation and mineralization, and fibrous

encapsulation of the intestinal and lymphatic lesions while rare in cattle are quite common in sheep (Howarth, 1932; Nisbet, Gilmour and Brotherston, 1962) and goats (Majeed, 1972).

In deer, gross intestinal and lymphatic lesions are mild and inconsistent, but comparable to those observed in sheep and goats. The post-mortem picture presented is frequently that of an emaciated carcase with a dull, rough, alopecic haircoat, dry skin, gelatinous body-fat depots, serous effusion into body cavities; and more severe cases may in addition have subserous oedema of the lower intestine and attaching mesentery (Bourgeois, 1940; Vance, 1961; Libke and Walton, 1975; Williams et al., 1983; Gumbrell, 1987). The intestinal mucosa may be slightly thickened (Libke and Walton, 1975) or just swollen and reddened in some parts (Bourgeois, 1940). Petechiation and extensive erosion of the mucosa have also been reported (Vance, 1961). Mesenteric lymph nodes and the draining lymphatic vessels may exhibit varying degrees of enlargement (Bourgeois, 1940; Vance, 1961; Libke and Walton, 1975; Gumbrell, 1987).

### 1.5.2 Histopathological changes

The inflammatory changes of paratuberculosis have been suggested to be analogous to those of leprosy (Buergelt et al., 1978), and despite involvement of different organ systems, the microscopic lesions are dominated by macrophages and giant cells. Buergelt and others (1978) described three morphologically distinct types of macrophages involved in the disease process in cattle. These are the elongate spindle-shaped macrophages arranged in syncytial-like pattern present within the intestinal mucosa as well as the mesenteric lymph nodes;

the polygonal macrophages with prominent foamy or vacuolated cytoplasm; and the well developed epithelioid cells with deep eosinophilic cytoplasm, distinct cell boundaries and eccentric oval hypochromic nuclei containing several nucleoli of various sizes. In earlier pathomorphological studies in goats, Majeed (1972) recognized two cell types involved in the paratuberculous process - the eccentrically nucleated paratuberculous cell which contained organisms, and the centrally nucleated epithelioid cell which was devoid of organisms. It seems that the paratuberculous cell of Majeed and the well developed epithelioid cell of Buergelt and others are analogous, and together with giant cells (Langhans type) they form the predominant pathognomonic cell types involved in the disease process.

The microscopic lesions of paratuberculosis may be tuberculoid (nodular or focal) or lepromatous (diffuse) granulomatous changes arising from invasion by the macrophages, epithelioid and giant cells (Chiodini et al., 1984a). Gilmour and co-workers (1965) found the focal occurrence of giant cells within Peyer's patches and within tips of intestinal villi to be the earliest specific changes of paratuberculosis. Jubb and co-authors (1985) describe the very early microscopic changes in the intestinal mucosa to be quite indefinite. Initially the lamina propria is diffusely, but loosely infiltrated by lymphocytes and plasma cells, and an unexpectedly large number of eosinophils. During this stage, there may also be a few epithelioid cells and macrophages, but the most characteristic and constant change is the loose infiltration of lymphocytes and plasma cells in the submucosa and in the walls of the submucosal and mesenteric lymphatics. As the disease progresses, the epithelioid cells and macrophages

increase in number to form multifocal aggregates in the villous tips while the other infiltrating elements are proportionately reduced. As more epithelioid cells, macrophages and giant cells progressively fill the lamina propria, the multifocal aggregates coalesce consequently broadening, shortening and distorting the villi to give them a club-shaped appearance. The villous lacteals are prominently dilated and cryptal glands distended. The pressure from the cell infiltrate in the villous and mucosal lamina propria combined with the lacteal and cryptal gland expansion result in the compression and obliteration of many of the intestinal crypts. As the invading cells further increase, they penetrate the muscularis mucosa to congregate in the submucosa. This gives rise to an extensive syncytium of typical epithelioid cells which is responsible for the gross thickening of the intestinal wall. Focal aggregates or diffuse infiltration of lymphocytes and giant cells are almost invariably present in the cellular masses. Foci of necrosis sometimes do occur within the cell masses in cattle, but caseation and calcification are rare. This feature generally distinguishes paratuberculosis in cattle from bovine intestinal tuberculosis. The Peyer's patches are usually hyperplastic and invested by the invading cells (Buergelt et al., 1978). The intestinal subserosa may become oedematous and more cellular. The intestinal lesions usually diminish in intensity towards the duodenum and the rectum.

Lymphatic histopathological changes include granulomatous lymphadenitis and proliferative endo- and peri- lymphangitis. In the early stages of the inflammatory process, lymphatic walls may be infiltrated by lymphocytes and plasma cells (Jubb et al., 1985). As the infection progresses, the small mononuclear cells are replaced by

macrophages and epithelioid cells to form granulomatous plugs of the large mononuclear cells which can project into the lumena of the vessels enough to cause obliteration. Plugs projecting over the outer surface of the vessels are responsible for the beaded appearance of the mesenteric lymphatics. The granulomas frequently show some necrosis. In the associated lymph nodes, macrophages, epithelioid and giant cells may initially loosely infiltrate and aggregate in the subcapsular sinuses. As the invasion progresses, the granulomatous cells infiltrate the paracortical areas to form follicular or diffuse patterns (Buergelt et al., 1978; Jubb et al., 1985) The invading cells may ultimately replace much of the cortex, but the medulla is usually devoid of these cells and organisms, with oedema being the most pronounced change. Lymphoid granulomatous changes have also been described on some occasions in the tonsils, retropharyngeal and other carcase nodes (Jubb et al., 1985).

Ziehl-Neelsen (ZN) stained sections of the intestinal and lymphatic tissue usually show AFB in the macrophages, epithelioid and giant cells, although on some occasions there may be no demonstrable organisms (Hallman and Witter, 1933).

The basic cellular response to M. ptbc infection in sheep (Stamp and Watt, 1954) and in goats (Levi, 1948; Majeed, 1972) does not differ from that of cattle, except that tubercle-like foci with necrosis, calcification and reactive fibrosis in the intestinal mucosa, submucosa and subserosa are not uncommon. The draining lymphatic vessels and nodes may also be involved with even more severe changes.

The microscopic lesions of paratuberculosis described in deer are quite inconsistent. Again as in cattle, granulomatous enteritis, lymphadenitis and lymphangitis are the prominent histopathological changes. Lymphoid granulomatous lesions have also been reported by previous investigators (Libke and Walton, 1975; Temple, Muscoplat, Thoen, Himes and Johnson, 1979; Chiodini and Van Kruiningen, 1983a; Buxton, 1987; Gumbrell, 1987). However the outstanding features of necrosis and calcification described in ovine and caprine paratuberculosis have also been reported in deer (Williams et al., 1983; Jørgensen and Jørgensen, 1987). It is not clear whether this inconsistency of pathological lesions observed in deer is due to variation in the characteristics of the infecting strains of M. ptbc or due to differences between individual deer in susceptibility to the infection.

Microscopic lesions in organs other than the intestinal and lymphatic tissue have been described in advanced cases of paratuberculosis in cattle, but the most specific changes are those found in the liver where aggregates of a few epithelioid cells, macrophages and lymphocytes scattered in the lobules may be observed (Mathews, 1930; Buergelt et al., 1978). Harding (1957, 1959) described microscopic lesions in the liver, lung and uterus in goats and small laboratory animals. In deer, disseminated microscopic lesions outside the intestines and associated lymphatic system have also been reported (Williams et al., 1983; Gumbrell, 1987; McKelvey, 1987).

Non-specific degenerative changes have also been described in cases of paratuberculosis in cattle, sheep and goats. Arteriosclerosis, fibrotic intimal thickening with calcification and plaques have been reported to occur in the abdominal aorta and the heart in cattle (Gifford, Eveleth and Gifford, 1942; Alibasoglu, Dunne and Guss, 1962; Simpson, 1966) and in goats (Majeed and Goudswaard, 1971). Fibrinoid and amyloid-like deposits in the small vessels of lymph nodes, adrenal glands and udder; pathological changes in the kidneys and aorta; and occasional neurologic lesions consisting of degenerative changes in the sciatic nerve and brachial plexus have been reported in goats (Nakamatsu, Fujimoto and Satoh, 1968). Fibrinoid and amyloid-like deposits in the small vessels of lymph nodes in goats were also reported by Paliwal and Rajya (1982). However Angus and Gilmour (1971) did not find comparable lesions in sub-clinical stages of paratuberculosis in sheep. In severe cases of paratuberculosis in goats, Majeed and Goudswaard (1971) reported kidney changes which consisted of fibrotic thickening with deposition of amyloid-like substance of walls of glomerular capsules, but no AFB were detected. Secondary endocrine lesions have been reported in goats (Rajan et al., 1980).

### 1.5.3 Ultrastructural changes

The relationship between M. ptbc and the cells of the lesions has been investigated by electron microscopy in goats (Majeed, 1972; Paliwal and Rehbinder, 1981) and in cattle (Bendixen et al., 1981; Kubo et al., 1983). The organisms were found located intracellularly in macrophages within phagosomes and phagolysosomes, and occasionally free

in the matrix of the macrophages in direct contact with the endoplasmic reticulum. Intact and degenerating bacilli were demonstrable in the intestinal macrophages under all the circumstances described.

1.5.4 Histochemical features

In goats, Majeed (1972) found a higher enzyme biochemical activity in organism-containing paratuberculous cells than in epithelioid cells which did not contain organisms. The paratuberculous cells contained acid phosphatase, aryl sulphatase, beta-galactosidase, non-specific esterase, glucose-6-phosphatase dehydrogenase, lactate dehydrogenase and succinic dehydrogenase, but alkaline phosphatase and 5-nucleotidase were not present whereas in the epithelioid cells, only acid phosphatase and aryl sulphatase were present. Paliwal, Rajya and Krishna (1984a) found an inverse relationship between acid phosphatase activity and number of M. ptbc in the macrophages, but the other enzymes reported by Majeed were found in the small intestine and contiguous lymph nodes irrespective of the presence or absence of the organisms.

Observations by Lepper and Wilks (1980, 1988) indicated a close association of intracellular iron with M. ptbc in macrophages of some granulomatous intestinal lesions in cattle, sheep and goats. Immunological studies of ileal granulomas (Momotani, Furugouri, Obora, Miyata, Ishikawa and Yoshino, 1986) and mesenteric lymph nodes (Momotani, Whipple and Thiermann, 1988) in bovine paratuberculosis have shown the granulomas to contain ferritin (FT), lactoferrin (LF) and small amounts of transferrin (TF). These findings suggest that FT

and LF may be important *in vivo* sources of iron for *M. ptbc*, and the increase in FT and LF in the granulomas may be related to the inflammatory hyposideraemia associated with the disease.

### 1.5.5 Haematological changes

In haematological studies of clinical paratuberculosis in cattle (Allen, Berrett and Patterson, 1967) and sheep (Reddy, Sriraman and Rao, 1982), the mean values for total erythrocyte (RBC) and leucocyte (WBC) counts, packed cell volume (PCV), haemoglobin (Hb) and mean corpuscular volume (MCV) did not differ significantly from non-infected animals, but individual cases had low RBC counts and Hb values. However the cells were normocytic and normochromic. As suggested in earlier work by Stewart, McCallum and Taylor (1945), the decrease in RBC and Hb values in affected sheep may be due to inadequate nutrients for haemopoiesis. For WBC counts, the neutrophil percentage tended to be higher, and the lymphocyte percentage lower than in normal animals; but when the animals were *in extremis*, the neutrophil:lymphocyte ratio was reversed, and an accompanying eosinopaenia was present which may reflect the presence of increased numbers of eosinophils in the intestines. In other earlier haematological studies (Rankin, 1955; Rajya and Singh, 1959), no change in erythrocyte sedimentation rate (ESR) was observed.

### 1.5.6 Biochemical changes

Changes in blood biochemistry of animals infected with *M. ptbc* are non-specific and they occur only late in the clinical disease. Measurement of plasma levels of 24 enzymes in clinical cases of

paratuberculosis in cattle showed virtually no enzyme abnormality despite the chronic enteritis and severe muscle wasting (Patterson and Allen, 1972). Due to the close association between the infecting organisms and intestinal macrophages, the activities of the lysosomal enzymes acid hydrolase, acid phosphatase, aryl sulphatase and beta-glucuronidase would be expected to be elevated in clinical paratuberculosis during the process of raised phagocytosis, but only the activity of beta-glucuronidase was significantly elevated. The other lysosomal enzymes were only slightly raised (Patterson et al., 1967).

Patterson and Sweasey (1966) reported elevation of serum glycoprotein concentration , and Patterson, Allen, Berrett, Ivins and Sweasey (1968) found a relative increase in alpha-globulins. Serum proteins, particularly the albumin content, was found to be reduced. This reduction was postulated to be associated with a slightly expanded extracellular water compartment, and a decrease in plasma volume explaining the dependent oedema in affected animals. Patterson and co-workers (1967) and Allen (1971) found a massive loss of plasma proteins across the gut wall in cattle and sheep sufficient in either species to account for most of the body weight loss. Amino acid malabsorption as reported by Patterson and Berrett (1968) may also contribute to the weight loss. Changes in muscle protein are those associated with wasting (Patterson et al., 1968).

Sodium retention and a corresponding potassium and chloride loss have been observed in clinical paratuberculosis (Patterson et al.,

1968). Reddy and others (1982) reported that the mean calcium, phosphorus and magnesium serum levels were significantly lowered in sheep suffering from paratuberculosis.

## 1.6 Immunity

Immunological responses in paratuberculous animals are not well understood, but it is clear that the thick, impervious cell wall and the intracellular residence of M. ptbc play an important role in protecting the organism from the natural and immune defence mechanisms of the host. Animals infected with M. ptbc may express the entire spectrum of immune responsiveness from CMI response to the humoral response (Bendixen, 1978); but the immunological protection involved is predominantly cell mediated. Initially there is a CMI (tuberculoid) response reflected as DTH, and later on as the disease progresses towards the lepromatous stage, the CMI response gradually wanes and a humoral response predominates in which circulating antibodies are detectable. Ultimately a state of anergy may be reached in which neither CMI nor humoral immunity can be detected. These immunologic responses occur independent of clinical signs, and may appear at any time during the clinical spectrum. However Larsen (1972) suggested that the CMI corresponded with the pre-clinical stage of the disease; and the humoral phase with the clinical period. Although in some animals, an overlap of the responses may be demonstrable throughout the active course of the disease (Larsen, 1972), generally there is an inverse relationship between the CMI and humoral immunity. In the review by Chiodini and co-authors (1984a), the CMI response is considered to be an attempt by macrophages to arrest the offending

organisms, and the humoral response to be triggered by the release of the organisms by the dying macrophages. Anergy during chronic paratuberculosis has not been thoroughly investigated, but it has been suggested that it results from the release of soluble factors from the macrophages. A humoral factor which suppresses lymphocyte transformation *in vitro* has been described in animals chronically infected with *M. ptbc* (Davies, Corbeil, Ward and Duncan, 1974).

## 1.7 Diagnosis

The diagnosis of paratuberculosis in an individual animal is difficult except at post-mortem examination. It is hampered by lack of accurate and sensitive tests to identify all infected individuals, and many of the tests produce significant numbers of false positive and false negative results. This feature is not unique to paratuberculosis, but rather common to most mycobacterial infections (Chiodini *et al.*, 1984a). Presumptive diagnosis in a herd of known paratuberculosis status may be quite easy. New cases can be identified by a combination of individual history and clinical examination (Gilmour, 1976). In cattle, the presence of chronic wasting and/or intractable diarrhoea in adults, and a reduction in milk production may lead to a correct diagnosis in a large number of cases. However the disease needs to be differentiated from other causes of wasting and diarrhoea either in a herd or in an individual animal. Such causes among others include general malnutrition, copper and cobalt deficiency, molybdenum poisoning, gastro-intestinal parasitism (e.g. ostertagiasis, fascioliasis and coccidiosis), malignant catarrhal fever, yersiniosis, bovine viral diarrhoea/mucosal disease,

rinderpest, salmonellosis, hepatic fibrosis, pyelonephritis and traumatic reticulo-peritonitis (Blood et al., 1983, Jubb et al., 1985). Definitive diagnosis of M. ptbc infection requires identification of the organism from suspected individuals, although immunological tests, and pathological examination are also employed for the detection of the infection.

### 1.7.1 Identification of M. paratuberculosis

#### 1.7.1.1 Microscopic examination

Microscopic demonstration of M. ptbc in faecal smears and rectal scrapings stained by the ZN technique is a widely used diagnostic method recommended by several investigators (Doyle, 1956; Cunningham and Gilmour, 1959; Hole and Maclay, 1959; Ringdal, 1960; Merkal, 1970; Desmecht, 1977) particularly in identifying shedders within an infected herd. Other alternative stains such as auramine (Rowlatt and Corner, 1981) and toluidine blue (Tamasi, 1981) have also been used, but there is no evidence of any significant advantage over the ZN stain.

The finding of clumps of small slender AFB either free or in macrophages, or great numbers of single rods of a morphology consistent with that of M. ptbc is considered diagnostic. However the drawback of microscopic examination of smears is the occurrence of false positive results which may arise from the presence in faeces of large saprophytic AFB such as the hay bacillus M. phlei (Gilmour, 1960; Merkal, 1970) and other AFB present in waters and the environment (Beerwith, 1973). On microscopic examination of faecal smears, Merkal, Larsen, Kopecky and Ness (1986b) found organisms which resembled M. ptbc in 91% of culture positive cows and in 83% of

necropsy positive cases, however 76% of the culture and necropsy negative cows had organisms in faecal smears which resembled, but were not M. ptbc. Various fungi are also acid-fast and may appear as numerous acid-fast fragments in faecal smears from animals fed mouldy hay or silage (Chiodini et al., 1984a).

Rectal scrapings are of limited value as they are positive only in longstanding cases (Doyle, 1956); and according to Larsen (1972), only 25% of the animals infected with the Johne's bacillus have lesions extending to the rectum. While a positive result by microscopy is very suggestive of a diagnosis of paratuberculosis, failure to demonstrate organisms does not merit a negative diagnosis because the organisms may be shed in the faeces intermittently or be too few to be detected (Larsen, 1972; Johnson, Muscoplat, Larsen and Thoen, 1977b). Repeated faecal examination is necessary since single examination may miss infected, non-shedding individuals. Rankin (1958b) reported finding M. ptbc in only 30% of infected animals on a single examination.

1.7.1.2 Cultural examination of faeces

Faecal culture is considered to be the most accurate and preferred means for the diagnosis of paratuberculosis (Merkal et al., 1968b; Thoen and Muscoplat, 1979). Comparative studies of some widely used diagnostic tests for paratuberculosis showed the results of faecal culture to correspond well to results of microscopic and cultural examination of intestinal mucosa of animals disposed of for the disease (Merkal et al., 1968b). From faecal culture, the organism could be detected many months before the appearance of clinical symptoms, and vaccinated animals shedding the organism can also be identified.

Merkal, Kopecky, Larsen and Thurston (1964) and Merkal (1970) recommended the use of faecal culture for screening herds of known paratuberculosis status; and Merkal (1973) considered the test to be the most suitable in detecting infection in apparently healthy cattle in known infected herds.

However the practical value of cultural results is seriously impaired by the slow growth of the organism. Thus a rapid diagnosis cannot be obtained to allow early disposition of the animal before costly non-specific therapy is initiated or before progressive wasting diminishes the animal's salvage value (Sherman, Markham and Bates, 1984). Furthermore faecal culture is not simple. Despite advances in culture methods for in vitro growth of M. ptbc, the recovery of the organism from faeces may be adversely affected by the decontamination process and contamination of the cultures.

### 1.7.2 Immunological methods

Generally the use of immunological methods for the detection of paratuberculosis in individual animals is of limited value, but on a herd basis, they can be used with success in differentiating infected from non-infected herds and in obtaining the prevalence of infection within a herd. A herd which does not contain reactors is probably free from the infection, but the presence of reactors does not necessarily mean that a herd has the disease since the immunologic response only provides evidence of previous exposure to M. ptbc or closely related organisms (Johnson, Muscoplat, Larsen et al., 1977b). Merkal (1973) and Thoen and Muscoplat (1979) subjected many immunological tests to critical evaluation. They found that all the tests used demonstrated

either diagnostic unreliability with an unacceptable frequency of false positive and false negative results or problems of practical application.

False positive immunological responses have been shown to occur as a consequence of cross-reactions from infections with other mycobacteria (Hagan and Zeissig, 1929; Chandler, 1956; Grange, 1980; Wilks, Taylor, Russell and Thomas, 1981; Camphausen et al., 1988), Corynebacterium spp (Larsen and Johnson, 1947; Goudswaard and Terporten-Pastoors, 1972; Gilmour and Goudswaard, 1972; McKenzie and Ward, 1981), Nocardia (Ridell, 1977), species of Actinomyces, Dermatophilus and Streptomyces (Chiodini et al., 1984a) and Blastomyces spp (Nain, Chandiramani, Kulshreshtha and Chugh, 1985). The cross-reactions are due to the crudeness of the antigens used for the tests which have antigenic determinants common to most organisms of the order Actinomycetales (Chiodini et al., 1984a). Positive immunologic reactions can also be encountered in completely recovered and vaccinated animals (Merkal, 1984).

On the other hand, false negative reactions can occur as a result of antigen masking factors (Sigurdsson, 1945, 1946, 1947a, 1947b) or tolerance and anergy. The failure to respond to antigen has been shown to occur most often during terminal stages of the clinical disease (Stuart, 1965a), however it may also be present at any stage during the chronic infection (Chiodini et al., 1984a). CMI reactivities have been found to be more prone to tolerance than humoral responses (Davis, Dulbecco, Eisen and Ginsberg, 1980). Thus an animal which is tolerant may exhibit a humoral response, but fail to show a CMI reaction. A

clinically healthy animal which had previously responded to antigen, but fails to respond to subsequent testing may be anergic but infected or may have completely recovered from the infection.

Immunologic tests for paratuberculosis can be divided into two categories. Allergic (DTH) tests which detect CMI responses and serologic tests which detect circulating antibody (humoral immune responses). DTH reactions are most rapidly induced, but disappear earlier than humoral responses (Merkal, Larsen, Kopecky, Kluge, Monlux, Lehmann and Quinn, 1968a). With heavy exposure to infection, most cattle have been found to develop a DTH reaction by nine months after exposure (Stuart, 1965a), whereas in less heavily infected cattle (Gilmour, 1965), and with more intermittent exposure as occurs in the field (Reinders, 1963), DTH reactions have been found to develop later and less regularly. For humoral responses, infected cattle have been reported to take about two years or more to mount an antibody response against M. ptbc which may disappear after a period of about 4-5 years as the animals develop anergy (Chiodini et al., 1984a). In the humoral response, precipitins usually appear earliest, followed soon afterwards by inhibition of haemagglutination (HA) and haemolysis (HL) and complement fixing antibodies (Merkal, Larsen, Kopecky, Kluge et al., 1968a).

1.7.2.1 Allergic tests

(a) In vivo allergic tests

Intravenous (i/v)- and intradermal (i/d)- johnin/tuberculin tests are the traditional in vivo allergic tests used in the field to provide indirect evidence of infection with mycobacteria.

(1) Intravenous johnin/tuberculin test

The i/v administration of johnin antigen (Hastings, Beach and Hadley, 1918) was the first method used for the diagnosis of paratuberculosis. Hagan and Zeissig (1933) used avian tuberculin purified protein derivative (PPD) instead of the johnin antigen. A positive reaction is indicated by a rise of body temperature of 1.5°C and above and/or a change in the neutrophil:lymphocyte ratio greater than 2:1 six hours after injection of the antigen (Allen et al., 1967; Kopecky, Booth, Merkal and Larsen, 1971). The latter parameter is considered to be more accurate than the temperature rise. The test has been reported to provide greater accuracy in differentiating clinical paratuberculosis from other causes of chronic diarrhoea, but it is unable to detect lightly infected animals (Larsen and Kopecky, 1965); and it is tedious because of the need for repeated visits to the animal to measure the temperature and to collect blood for differential counts.

(2) Intradermal johnin/tuberculin test

The i/d injection of johnin or avian tuberculin PPD is widely used for screening herds for paratuberculosis (Larsen, 1951; Larsen, Vardaman and Merkal, 1963). A positive i/d test is indicated by an increase in skin thickness of five mm or greater 48-72 hours after injection of the antigen. Larsen and co-workers (1963) found that repeated reactors to the test frequently became non-reactors and some cattle reacted intermittently. Thus not all infected animals can be detected by a single test. Larsen and co-workers (1963) suggested that

those animals which were sensitive to the antigen for several years are more likely to develop the clinical disease than those which have reverted to the negative status.

Triple i/d allergic tests using avian and bovine tuberculins and johnin each injected separately three times at 48-72 h intervals were shown to improve the sensitivity and specificity of the skin test (Cellarius, 1971; Crăciunescu, Ionică, Miciora and Dăcilă, 1982).

Comparative i/d tests (Ritchie, 1953; Lesslie, Hebert and Frerichs, 1976) are also widely used to distinguish M. ptbc and M. avium infections from tuberculosis. Avian and mammalian (bovine) tuberculin PPD's are the antigens commonly used in practice. The sensitivity and specificity of the comparative skin tests can be improved by measurement of induration diameter in addition to the skin thickness (Lesslie, Hebert, Burn, MacClancy and Donnelly, 1975). However interference or interactions between the several antigens may occur in the comparative i/d tests.

Like the i/v test, repeated farm visits are necessary for subsequent skin measurements. While a positive i/d test is only indicative of exposure to the organism, a negative result does not necessarily mean the absence of infection since, as recorded above, with progression of the disease, the animals may become immunologically unresponsive due to anergy (Davies et al., 1974).

(b) In vitro allergic tests

In vitro allergic tests for detection of CMI responses have been described as possible laboratory diagnostic tests for paratuberculosis

(Allen, Hebert and Patterson, 1968; Aalund, Hoerlein and Adler, 1970). These include leucocyte migration and leucocyte migration inhibition tests, and several tests for lymphocyte stimulation and transformation.

(1) Leucocyte migration agarose test

The leucocyte migration agarose test (Aalund et al., 1970) was used by Bendixen (1977) to detect M. ptbc infection in a known infected herd. He considered the test to be of no diagnostic value because reactors were found even in cattle that had no evidence of infection at post-mortem examination. This response was suggested to be a result of prior exposure to the antigen without persistent infection.

(2) Leucocyte migration inhibition test

The leucocyte migration inhibition (LMI) test (Kataria, Somvanshi and Paliwal, 1985) can be adopted for diagnosis of paratuberculosis, but its use for detecting pre-clinical cases of the disease has yet to be evaluated.

(3) Lymphocyte stimulation/transformation test

A number of tests of lymphocyte stimulation and transformation (LT) have been developed (Alhaji, Johnson, Muscoplat and Thoen, 1974; Muscoplat, Chen, Johnson and Alhaji, 1974; Johnson, Muscoplat, Hoeffling and Thoen, 1977a). Buergelt, Hall, Merkal, Whitlock and Duncan (1977) and Buergelt, De Lisle, Hall, Merkal and Duncan (1978) compared the LT test with some other diagnostic methods of paratuberculosis (histopathology, complement fixation, gel precipitation and faecal culture) and considered the test to be of value as an indicator of the

spread of infection within a herd but repeated tests are necessary. Johnson and others (1977a), De Lisle (1979b) and De Lisle and Duncan (1981) demonstrated specific immunological reactivity to johnin even in minimally infected animals. The LT test was found to be the most accurate of the commonly used tests for the diagnosis of paratuberculosis in deer with the added advantages of there being no need to restrain the animals a second time, and that several antigens can be used without interference or interaction between the antigens, as may be the case with the skin test (Temple et al., 1979). Modifications of the LT test (Milner, Wilks and Borland, 1981) improved its diagnostic potential.

However the test has its disadvantages. It is complex and expensive, and hence considered unsuitable for large-scale screening of large numbers of individuals or whole herds (Van Reenen, 1988). Heavily infected animals show considerable variation in their LT responses (De Lisle and Duncan, 1981); and not all carriers of the infection can be detected (Wentink, Rutten, Jaartsveld, Zeeuwen and Van Kooten, 1984). Furthermore the status of infection in individual animals cannot be determined especially in detecting pre-clinical infections and in differentiating infected from recovered and vaccinated individuals (Bendixen, 1977; Hintz, 1981); and significant false positive and false negative results have been encountered in domestic ruminants (De Lisle, 1979b) and deer (Williams, De Martini and Snyder, 1985).

1.7.2.2 Serological tests

(1) Complement fixation test

The complement fixation (CF) test (Twort and Ingram, 1913) is the traditional and most widely used serological test for the detection of infection with M. ptbc. The test is of value only in detecting advanced cases of infection (Bang, 1914; Hagan and Zeissig, 1933; Larsen et al., 1963; De Lisle et al., 1980). Rankin (1958a) evaluated its sensitivity and specificity and found it to be almost always positive when the disease was advanced and clinical signs evident, but frequently it failed to give positive reactions in the sera of carrier animals, and 10-20% false positive reactors were encountered in herds where neither disease nor infection existed. Gilmour and Goudswaard (1972) and Goudswaard and Budhai (1975) found some of the false positive results in cattle to be due to cross-reactions from infection with Corynebacterium renale. To improve specificity and sensitivity of the test, Goudswaard and Budhai recommended the use of parallel C. renale and M. ptbc antigens with specificity being indicated by the test giving the higher titre. Results of the CF test in deer, as in the other species, further indicate that it is not useful in detecting pre-clinical paratuberculosis (Williams et al., 1985).

(2) Conglutinating complement absorption test

A conglutinating complement absorption (CCA) test has been reported to be more sensitive than the CF test giving a 2-3 fold higher titre (Kulshrestha, Paliwal and Krishna, 1984).

(3) Fluorescent antibody test

The indirect fluorescent antibody (FA) test (Gilmour and Gardiner, 1969) is reported to be more efficient than the CF test earlier in the course of infection and equally good in confirming the disease when lesions are advanced (Gilmour and Angus, 1976b). In experimentally infected rabbits, Gilmour (1971) showed the test to be specific in detecting antibody to M. ptbc. Unlike the CF test, the FA test does not show cross-reactions with C. renale (Gilmour, 1976). In cattle experimentally infected with M. ptbc and with M. avium, Gilmour and Angus (1976a) demonstrated that the FA test was more specific than the CF test, while in detecting antibody to M. ptbc, the FA test was found to be as sensitive as the CF test (Goudswaard, Gilmour, Dijkstra and Van Beek, 1976). The use of the FA and CF tests together was considered to be of value in detecting sub-clinical infections (Goudswaard et al., 1976), however Abbas, Riemann and Behymer (1983) considered neither of the two tests to have acceptable sensitivity and specificity for detecting sub-clinical cases of paratuberculosis. Interestingly in naturally infected goats, Paliwal, Rajya and Krishna (1984b) found the FA test to be a better diagnostic tool at all levels of infection especially in pre-clinical stages than microscopic examination of faeces and the i/d johnin test. However they recommended repeated herd testing due to variation in the serological response during successive tests.

(4) Haemagglutination and haemolysis tests

The haemagglutination (HA) test (Larsen, Vardaman, Kopecky and Merkal, 1965; Larsen et al., 1963) and haemolysis (HL) test (Goudswaard, 1971) have been found in cattle to pick specific antibody to M. ptbc, but the antibody response did not appear to correlate closely enough with the finding of typical AFB at post-mortem examination. However the tests are considered to be of diagnostic significance in goats (Goudswaard, 1971). The HA and HL tests were found to be relatively more insensitive than the CF and FA tests during the pre-clinical stages of paratuberculosis (Goudswaard et al., 1976), but may be of value as confirmatory test in the presence of reactions to the CF and FA tests.

(5) Agar gel immunodiffusion test

Agar gel immunodiffusion (AGID) test is a widely used serological test for the diagnosis of paratuberculosis (Merkal, 1973). In sheep, the test has been reported to reflect the progress of M. ptbc infection more accurately than the CF and HA tests (Merkal et al., 1968b). The antibody response was found to increase as the disease advanced, and a strong correlation was established between antibody levels detected and the degree of faecal excretion of the organisms. This was reflected by an increase in the number and density of precipitation bands over time. Like the CF test, the AGID test lacks diagnostic sensitivity for detecting sub-clinical infections (Merkal et al., 1968b; Buergelt, De Lisle, Hall et al., 1978). On the basis of post-mortem confirmation of clinical paratuberculosis in goats (Sherman

and Gezon, 1980; Thomas, 1983), and in cattle with signs of diarrhoea and/or weight loss (Sherman, Markham and Bates, 1984), the AGID test has shown diagnostic sensitivity and specificity equal to that of bacterial culture of faeces. The test is also easier to perform, less expensive and more rapid than bacteriological culture. However cross-reactions with precipitation antibodies to C. renale in cattle (Ridell, 1977) and M. butyricum in goats (Sherman and Gezon, 1980), resulting in non-specific precipitation lines have been encountered. Preabsorption of the sera with M. phlei has been reported to improve the specificity of the test (Merkal, 1984).

(6) Counter-immunoelectrophoresis

By counter-immunoelectrophoresis (CIE) (Muhammed, Tadayon and Cheema, 1978), antibody to M. ptbc can be detected rapidly. The test has been shown in sheep and goats to be at least of higher sensitivity than the AGID test (Muhammed and Ivoghli, 1983; Brooks, Robertson, Corner, Samagh, Garcia, Turcotte and Duncan, 1988).

(7) Sandwich solid phase radioimmunoassay

By sandwich solid phase radioimmunoassay, Worsaae (1978) was able to detect antibody to M. ptbc in infected animals before their bacterial load was high enough to be detected by faecal culture, implying a higher sensitivity than many of the diagnostic tests for paratuberculosis.

(8) Enzyme-linked immunosorbent assay

The enzyme-linked immunosorbent assay (ELISA) (Jørgensen and Jensen, 1978; Thoen and Bruner, 1982; Abbas, Riemann and Lonnerdal, 1983), is replacing the other serological tests for paratuberculosis. Previous ELISA's like the other serological tests were not free from problems of non-specificity. Great improvements in the specificity of the test have been made by preparation of more specific antigens (Yokomizo, Merkal and Lyle, 1983), and absorption of the sera with a suspension of M. phlei to remove the non-specific cross-reacting antibodies to antigens of Corynebacterium, Nocardia, M. bovis and most non-group 111 mycobacteria (Yokomizo, Yugi and Merkal, 1985; Milner, Lepper, Symonds and Gruner, 1987). The ELISA method has been found to usually detect infected animals earlier than AGID and CF tests which are not positive until high numbers of the organism are being excreted. However the potential drawback of ELISA is that it may still be positive in animals that had been lightly infected and recovered (Merkal, 1984), and vaccinated individuals, thus weakening the power of the test for diagnosis of sub-clinical infections (Sherman, 1985).

(9) Dot immunoblotting assay

The dot immunoblotting assay (DIA) (Tsai, Hutchinson and Zarkower, 1989) is of comparable efficacy to the ELISA test in detection of antibody to M. ptbc and has the advantage of simplicity, rapidity and cheapness and the potential for field use.

1.7.2.2 Indirect immunoperoxidase test

Nguyen and Buergelt (1983) used an indirect immunoperoxidase test for detection of M. ptbc antigen in formalin fixed tissue. They found the test simple and considered it valuable in routine screening for the antigen in biopsy samples of rectal mucosa or other infected tissues. However its validity and practical use in a field situation requires critical evaluation.

1.7.3 Pathological examination

Post-mortem diagnosis of paratuberculosis can be achieved by demonstration of the characteristic macroscopic intestinal and mesenteric lymph node lesions in animals which have died or have been killed in an advanced stage of the disease (Rankin, 1958b), but in sheep and goats, and in some cases in wild ruminants, gross lesions are often insufficient to suggest paratuberculosis.

A histopathological examination of serial sections of the lower intestinal tract and associated lymph nodes is often necessary to demonstrate the characteristic microscopic changes of the infection.

To obtain more certain identification of the organism, smears and cultures can be made from the intestinal mucosa and lymph nodes for microscopy and isolation.

1.7.4 Lymph node biopsy

Laparotomy to excise the ileocaecal lymph node for culture and histopathology can provide early and fairly accurate results for the detection of M. ptbc infection (Rines and Langham, 1964; Pemberton,

1979). However the surgical technique is time consuming and invasive, and the surgical scar may reduce the salvage value of the animal at slaughter (Sherman, 1985).

### 1.7.5 DNA probes

In recent years DNA probes have been developed for identification of M. ptbc (McFadden, Butcher, Chiodini and Hermon-Taylor, 1987; Hurley, Splitter and Welch, 1989; Moss, Green, Tizard, Malik, Visuvanathan, McFadden and Hermon-Taylor, in press; Murray, Moriarty and Scott, 1989) and other mycobacteria (Roberts, McMillan and Coyle, 1987; Musial, Tice, Stockman and Roberts, 1988). The test is specific and possesses the advantage of rapidity over the culture method. When combined with polymerase chain reaction (PCR) amplification (Vary, Andersen, Green, Hermon-Taylor and McFadden, 1990), very small numbers of bacteria, below detectable limits by culture, can be detected in faecal, tissue and other samples. However the test may be too expensive for routine use.

## 1.8 Treatment

In attempts to eliminate paratuberculosis by chemotherapy, over 100 antimycobacterial drugs have been investigated by several workers (Merkal, 1984). M. ptbc has been found to be refractory to penicillin, chloramphenicol, oxytetracyclines, sulphones and thiosemicarbazones (Larsen, Vardaman and Groth, 1950; Larsen and Vardaman, 1952; Smith, 1954a). Among other drugs tried are streptomycin (Larsen et al., 1950), viomycin, 4:4' diamino diphenyl sulphone and isonicotinic acid hydrazine (Larsen and Vardaman, 1952, 1953), isoniazid (Rankin, 1953),

rimino phenazine B663 (G 303220) (Gilmour, 1966, 1968) and clofazimine (Merkal and Larsen, 1973), but none of the drugs has shown success in eliminating the infection. In vitro experiments have yielded promising results of susceptibility of M. ptbc to a number of the drugs tested, but they have proved ineffective when tried in vivo (Spicer, 1936; Michael, 1946; Rankin, 1953; Gilmour and Angus, 1971; Hintz, Merkal, Whipple and Lyle, 1983). The intracellular location and impervious cell wall of the organism are undoubtedly barriers to drugs. Treated animals often improve clinically, but on withdrawal of the drugs, they continue to shed the organisms and eventually succumb to the disease (Larsen and Vardaman, 1953; Rankin, 1953; Merkal and Larsen, 1973; Whitlock, Divers, Palmer, Acland, Bruce and Tulleners, 1983). The use of isoniazid alone and in combination with streptomycin in calves did not prevent establishment of the infection (Rankin, 1955). The antituberculous drug B663 used for the treatment of leprosy and M. ulcerans skin ulcers, a drug which tends to concentrate in macrophages, was found to reduce the weight of infection in mice, but not eliminate it (Gilmour, 1966). In sheep (Gilmour, 1968; Angus and Gilmour, 1971; Gilmour and Angus, 1971), and in calves (Gilmour, 1970), B663 only delayed, but did not prevent establishment of the infection. Clofazimine (Merkal and Larsen, 1973; Whitlock et al., 1983) and amikacin sulphate (Merkal, 1984) are promising drugs, but the latter is extremely expensive. The effect of anti-inflammatory and immunosuppressive agents in the treatment of paratuberculosis has also been investigated, but such drugs appear to influence only the character of the infection and not to prevent development of the

lesions (Chiodini et al., 1984a). It has been suggested that the clinical disease being a terminal event, animals may be too severely affected to respond.

Chiodini and co-authors (1984a) are of the view that the only hope for chemotherapy against paratuberculosis is the development of new antituberculous drugs, but the cost of the drugs and the protracted course of treatment required may preclude the widespread use of chemotherapy in other than pedigree breeding stock.

## 1.9 Prevention and control

Unless it involves a single, newly introduced infected animal, paratuberculosis is usually a herd problem. Considering the epidemiology of the disease, the control of paratuberculosis is difficult. The lack of accurate tests for its diagnosis, together with the chronic insidious nature of the disease can make elimination of the infection from a herd a prolonged and sometimes unsuccessful task (Blood et al., 1983; Sherman, 1985).

Paratuberculosis can be prevented from entering herds which are free from the infection by avoiding introduction or contact with infected animals but once the infection has gained access into a herd, its elimination demands drastic and stringent management changes, and identification and disposal for slaughter of infected individuals (Sherman, 1985). Vaccination of young animals may be necessary to increase resistance of the residual herd.

Infected individuals in a herd can be identified for disposal by allergic and serological tests and faecal culture. However eradication programmes based on allergic and serologic tests are often accompanied by the needless loss of many uninfected (false positive) animals (De Lisle et al., 1980). The culture method is considered to be much more accurate (Merkal, 1973), but intermittent shedders are likely to be missed. The chances of picking up more shedders can be increased if faecal examination of the residual herd is carried out at least twice yearly. The "culture and cull" method has been reported to be effective in eliminating the clinical disease from herds, but results depend largely on the degree of contamination of the environment (Moyle, 1975).

Management changes aimed at breaking the cycle of faeco-oral transmission of M. ptbc require measures of general hygiene and sanitation to minimize faecal contamination of drinking water, pasture and feed. The management of young animals from birth to breeding age also demands special attention. In cattle, separation of the offspring from their dams soon after birth , and rearing them separately from one another and from adults for as long as practicable (Rankin, 1958b; Merkal et al., 1975), preferably up to breeding age (Sherman, 1985) has been recommended together with feeding pasteurized colostrum or hygienically collected colostrum from paratuberculosis negative dams on the first day of their life, and thereafter giving them commercial milk replacer or pasteurized milk (Merkal, 1984). All offspring of clinically infected dams should be disposed of as they are likely to be infected in utero or from the faeces of their dams at calving (Pearson and McClelland, 1955). Replacement stock should only be recruited from

certified paratuberculosis free herds. As sheep, goats and wild ruminants are frequently sub-clinically infected until late in the course of the disease, they should be reared separately from cattle. Stress and other predisposing and precipitating factors described in section 2.1.3 should best be avoided. Changes in management in an infected herd have been reported to be effective in lowering the number of clinical cases and carriers of the infection (Doyle, 1956; Hole, 1958; Ringdal, 1965; Larsen and Merkal, 1968); and when coupled with scrupulous culling programs, stringent management changes have been shown to be effective in accelerating elimination of the disease in a herd, and under such circumstances, vaccination may not be necessary (Merkal, 1984).

Although vaccination has not proved entirely effective in preventing all cases of paratuberculosis (Hore, McQueen and McKinna, 1971), adequate evidence is available that adjuvanted vaccines containing killed organisms (Sigurdsson, 1960; Larsen, Hawkins and Merkal, 1964; Larsen, Moyle and Himes, 1978) or live organisms (Stuart, 1965a) give protection against clinical paratuberculosis (Gilmour, 1976). Vaccination does not eliminate the infection in an affected herd, but it has been shown to reduce the number of clinical cases (Doyle, 1945, 1964; Spears, 1959), and the number of faecal excretors and weight of excretion of the organisms (Stuart, 1965a). Immunization operates via a CMI response which inhibits intracellular multiplication of the organisms (Gilmour, 1965; Larsen, 1973). Hence to be effective, a vaccine has to cause a hypersensitivity response (Gilmour and Brotherston, 1966; Gilmour, Singleton and Ross, 1969). Vaccination is non-beneficial in already infected animals (Blood et al., 1983). As

immunized animals are still capable of spreading the infection, they should never be allowed into herds free from the infection. Vaccines which contain killed whole bacterial cells have been found in cattle to have superior immunizing power compared to vaccines made up of fractionated cells (Larsen et al., 1978). One dose of vaccine given within the first month of life is recommended (Sherman, 1985). A major negative aspect of vaccination is that it sensitizes some of the animals to the tuberculin test, and it also induces false positive serological reactions to paratuberculosis (Larsen et al., 1978). However the immune reaction due to vaccination against paratuberculosis can be differentiated from tuberculosis in most cases by comparative tests (Stuart, 1962; Larsen, Merkal, Kopecky and Boothe, 1969; Wallace, Carriere, Diena and Greenberg, 1971; Alhaji et al., 1974). Vaccination has been recommended in heavily infected , but tuberculosis free herds, and in range cattle, where management schemes such as segregation are impracticable (Gilmour, Stuart and Lloyd, 1977). The vaccine has to be administered carefully. It can cause a severe granuloma in man (Bjornsson et al., 1971), and an unsightly draining abscess or persistent granuloma in animals (Huitema, 1968). If management changes are not implemented and heavy shedders removed from the herd, the environmental load of M. ptbc can become high enough to override the protective effects of vaccination and new infections will continue to occur (Wilesmith, 1982).

# CHAPTER 2

## General Materials and Methods

### 2.1 Detection of paratuberculosis in study animals

The methods used for detection of infection in live and dead study animals were as follows:

#### 2.1.1 Live animals

##### 2.1.1.1 Clinical examination

Infected animals were examined for evidence of clinical abnormalities.

##### 2.1.1.2 Faecal examination

To test for faecal excretion of M. ptbc, fresh faecal samples were examined microscopically and by culture. The methods used for microscopic examination of smears and culture are described in sections 2.2.2.1 and 2.2.1.2, respectively.

##### 2.1.1.3 Intradermal tuberculin test

A single i/d comparative tuberculin test following the method described by Lesslie and Hebert (1975) was used for testing for DTH. Avian tuberculin PPD (0.5 mg/ml) and bovine tuberculin PPD (1.0 mg/ml) (MAFF, Central Veterinary Laboratory, Weybridge) were the antigens used.

Using animal grooming clippers (Oster model A-5), an area of the skin (10 by 10 cm) was thoroughly clipped on both sides of the

middle third of the neck. The sites were inspected and palpated for the presence of skin lesions and scars prior to measurement of the skin and tuberculin injection. Skin fold thickness at the sites of injection was measured with engineer's vernier caliper (Scala, West Germany) and recorded before injection (0h) and 72 hours later. For consistency, the avian PPD was injected into the left and the bovine PPD into the right side. Using separate 1.0 ml disposable plastic syringes and 26G x 3/8" needles for the two antigens, 0.1 ml of the respective PPD was injected i/d into the site.

During the second (72 h) reading, the presence of a visible or palpable swelling and/or an increase in skin thickness of three mm or more between 0 and 72h were regarded as a positive reaction to the test.

2.1.1.4 Serological examination

A modified indirect ELISA based on the methods previously described by Burrells, Wells and Dawson (1979), Abbas, Riemann and Lonnerdal (1983) and Yokomizo and others (1983) was used for detection of antibody to M. ptbc.

(1) Blood collection and serum separation

About 10 ml of blood was collected by jugular puncture using a vacutainer needle and tube (Becton Dickinson, Meylan Cedex - France) and allowed to clot overnight at room temperature. Serum

was separated by centrifugation on a bench top centrifuge (Centaur 2; MSE Crawley Sussex) at 2000 rev/min (660xg) for 10 min and stored at -20°C until use.

(2) Antigen preparation

A crude antigen derived from lysed cells of an isolate of M. ptbc (M961) from a deer was prepared following a modification of the method described by Abbas and co-workers (1983).

The organism was grown on solid modified Dubos medium (described in section 2.2.2.1) in 175 $cm^2$ polystyrene tissue culture flasks (Nunclon; A/S Nunc, P.O. Box 280, Kamstrup, Denmark). After eight weeks of incubation at 37°C, the growth on the surface of the medium was harvested by scraping with a sterile bent pasteur pipette. Approximately 0.2 - 0.3 g (wet weight) of growth was obtained from one flask. The cells were washed in sterile normal saline solution (0.85% sodium chloride) and then suspended in three ml of sterile phosphate buffered saline (PBS; Appendix 1a). After lysis in a French pressure cell (AMINCO) at 10,000 p.s.i., the cell lysate was centrifuged at 3000 rev/min (1500xg) for 30 min to remove unbroken cells and debris. The supernatant (crude antigen) was then stored at -20°C until use. A protein estimation of the antigen was made by the BCA Protein Assay (Pierce Chemical Company, P.O. Box, Rockford, Illinois 61105, U.S.A.).

(3) ELISA procedure

An optimal antigen concentration of 10 µg/well and a serum dilution of 1/40, both determined by checkerboard titration, were used for the assay. Unless otherwise stated, working volumes were 100 µl/well. Three washes between steps were made in ELISA wash buffer (EWB) which was PBS (pH 7.4) containing 0.05% Tween 20 (Sigma Chemical Company, P.O. Box 4508, St. Louis, MO 63178 USA). For coating the plate, the antigen was suspended in ELISA coating buffer (ECB) pH 9.6 (Appendix 1b).

A polystyrene micro-ELISA plate (M129A; Dynatech) was washed and then coated by adding the antigen to each well except those in column 1 (blank) which were each filled with plain ECB. The plate was then sealed with a Titertek plate sealer (Flow Laboratories Ltd, Irvine, Scotland) and incubated for 18 hours at 4°C.

After the absorption stage, the plate was again washed before addition of serum. All serum dilutions were made in EWB. Plain EWB was added to the wells in the blank column and positive control serum doubly diluted (1/20 to 1/2560) to the wells (in duplicate) in columns 2 and 3. Negative control serum diluted 1/40 was added to the wells in column 4 and test sera also diluted 1/40 to the rest of the wells in duplicate. The plate was then sealed and incubated at 37°C for one hour.

After washing the plate, 1/200 dilution in EWB of alkaline phosphatase (AP) pig anti-sheep IgG prepared following the method described by Burrells and others (1979) was added to each well. The plate was then sealed and incubated at 37°C for one hour.

For the colour reaction, the plate was again washed and then AP substrate (p-nitrophenyl phosphate; PNPP; Sigma) at a concentration of 1.0 mg/ml in 10% diethanolamine (Sigma) buffer (pH 9.8) containing 0.5M magnesium chloride (BDH Chemicals Ltd, Poole, England) added. The reaction was allowed to continue for one hour at room temperature and further colour development stopped by adding 50 µl of 3.0M sodium hydroxide (FSA Laboratory Supplies, Bishop Meadow Road, Loughborough, LE11 ORG, England) to each well.

The plate was then read spectrophotometrically in an ELISA reader (Titertek Multiskan R; Eflab Oy, Helsinki, Finland) using a 405 nm filter. Median optical densities (OD) of the duplicate wells above 0.04, a cut-off absorbance determined previously by checkerboard titration of the positive and negative standard sera, were considered positive.

### 2.1.2 Dead animals

#### 2.1.2.1 Post-mortem examination

Carcases were first inspected for external lesions and then opened for gross examination of the internal organs. Particular attention was paid to the gastrointestinal system and its associated lymphatic tissues.

During the post-mortem examination, tissues were collected for histopathology and bacteriological examination following previously described methods (Nisbet et al., 1962; Gilmour et al., 1965). In recently killed animals, the mesenteric lymph nodes were collected aseptically. Three tissue pools, of two nodes each, representing

the nodes draining the distal, middle and proximal segments of the small intestine were taken. The nodes were halved; one part was used for culture and the other for histopathology. The small intestine was then separated from its mesenteric attachment and three one metre lengths representing its distal, middle and proximal thirds excised. For histopathology, two blocks, each approximately three cm long and including a Peyer's patch were collected. Additional material from any other areas showing macroscopic changes was also sampled. The remainder of the small intestine was collected for culture. In addition, tissue samples were taken for histopathology from the caecum, proximal and distal colon, rectum, forestomachs, oesophagus, tonsils (half), retropharyngeal (half) mediastinal, and other carcase nodes (prescapular and prefemoral) and selected sites in the liver, spleen, kidneys, lungs, heart and the reproductive organs. The remaining halves of the tonsils and retropharyngeal lymph nodes were used for culture.

2.1.2.2 Histopathological examination

Tissues for histology were fixed in 10% neutral formol-saline for at least 48 hours and after trimming, transferred into formol sublimate for secondary fixation. After the blocks were processed and embedded in paraffin, five µm sections were cut and stained routinely by Mayer's haematoxylin and eosin (H & E). Replicate sections were stained by the ZN method for demonstration of acid-fast organisms.

2.1.2.3 Bacteriological examination of tissues

Aseptically collected tissues were processed for culture following the procedures described in subsection 2.2.1.2 (1) and intestinal and other contaminated tissue samples following the procedures described in subsection 2.2.1.2 (2).

2.2 Isolation and identification of M. paratuberculosis

2.2.1 Isolation

2.2.1.1 Media

The basic media which were used for the investigations described in this thesis were the solid and liquid modified Dubos media (Smith, 1953) and Watson-Reid salt base (Watson, 1935).

(1) Modified Dubos medium

The modified Dubos medium is essentially the William Smith's medium improved by Brotherston and others (1961). It is a serum-containing medium. The solid form of the medium containing mycobactin (2 µg/ml) was used for the primary isolation and subculture of M. ptbc. This medium was selected because its antibiotic and antifungal constitutents prevented overgrowth by contaminants and, being transparent, it allowed early detection of colonies.

A litre of the solid medium was prepared from the following formula: 2.5g casamino acids (casein hydrolysate peptone 5; Gibco Limited, P.O. Box 35, Paisley, Scotland), 0.3g neutral

l-asparagine (Sigma), 2.5g anhydrous di-sodium hydrogen phosphate (FSA); 1.0g potassium di-hydrogen phosphate (BDH), 1.5g tri-sodium citrate (FSA), 0.6g crystalline magnesium sulphate (BDH), 25 ml glycerol (FSA), 50 ml of 1% solution Tween 80 (Sigma) and 15.0g bacteriological agar (Gibco).

Each salt was dissolved in turn with minimum heat in deionized distilled water (DW) and made up to 800 ml. Mycobactin J (Allied Laboratories, Inc, USA, P.O. Box 1063, Ames IA 50010)) was then added to a final concentration of 2 mg/l and the mixture heated to 100$^{o}$C by free steaming. After addition of 0.05g chloramphenicol (Sigma), the medium was sterilized by autoclaving at 10 p.s.i. (115$^{o}$C) for 15 min and then cooled to 56$^{o}$C in a waterbath. This was followed by addition of 200 ml of newborn calf serum (Gibco) inactivated by heating at 56$^{o}$C for one hour and the antibiotics penicillin (Benzyl penicillin sodium BP, 100,000 units; Glaxo Laboratories, England) and Amphotericin B (Fungizone, 500 mg; E.R. Squibb and Sons, Inc Princeton NJ 08540, USA). The medium was mixed thoroughly and then dispensed in 5-6 ml quantities into sterile screw capped bottles and allowed to set in a sloped position. The water of condensation was aspirated off the slants 24 hours before use. The bottles were then stoppered with cotton-wool plugs and incubated at 37$^{o}$C for the slopes to dry.

Non-mycobactin containing solid Dubos medium prepared in the same way was used for testing for mycobactin-dependence of the isolates of mycobacteria used in the studies.

A liquid form of the Dubos medium prepared from the same formula but without agar was used for the maintenance of M. ptbc and for growing cultures of the organism for molecular biological studies.

(2) Watson-Reid salt base

The Reid's salt base, an iron-enriched liquid medium was used for dilution of inocula. A litre of the base was prepared from the following formula: 5.0g neutral l-asparagine, 2.0g anhydrous potassium di-hydrogen phosphate, 1.0g crystalline magnesium sulphate, 2.0g ammonium citrate (BDH), 2.0g sodium chloride (FSA), 0.075g ferric ammonium citrate (Sigma), 10.0g glucose monohydrate (Koch-Light Laboratories Ltd, Harverhill, Suffolk, England), 60.0g glycerol and deionized DW made up to 1000 ml. After the ingredients had dissolved thoroughly, the pH of the mixture was adjusted to 5.6-5.8. The base was then dispensed in 100 ml volumes and sterilized by autoclaving at 121$^{o}$C (15 p.s.i.) for seven min. To avoid caramelization of glucose by overheating, the medium was removed from the autoclave as soon as safely possible and the containers spaced for rapid cooling.

2.2.1.2 Culture techniques

The following procedures were used for culture of samples with little or no contamination by other organisms and contaminated samples.

(1) Culture of samples uncontaminated by other organisms

The method described by Brotherston and others (1961) was used for culture of samples collected aseptically from mesenteric lymph nodes and other organs from recently killed animals.

Ten ml of sterile PBS was added to five g of tissue sample weighed in a sterile stomacher bag. The mixture was then homogenized in a Colworth Stomacher 80 (A.J. Seward, UAC House, Blackfriars Road, London) for two minutes. Two 10-fold dilutions ($10^{-1}$ and $10^{-2}$) in Watson-Reid salt base of two 1.0 ml aliquots of the homogenate were made; and from each dilution, 0.1 ml was inoculated onto the surface of one or more slants of dried, solid, modified Dubos medium with and without mycobactin. The slopes were then screw capped tightly and laid horizontally overnight at room temperature for the inoculum to spread over the surface of the slope and then incubated upright for at least 8-12 weeks at $37^{o}C$.

(2) Culture of contaminated samples

To prevent overgrowth by organisms other than mycobacteria, faecal, intestinal and other contaminated samples required a decontamination stage before inoculation onto the culture medium. Although several different decontamination procedures have been described by Whipple and Merkal (1983), the following modified procedures were used.

(a) Culture of intestinal and other contaminated tissue samples

Intestinal mucosa was prepared following the procedure described by Brotherston and others (1961). After opening and washing the intestinal wall with tap water, the mucosa was scraped off using the edge of a microscope slide. To each of 4 x 10g aliquots of the mucosal scrapings weighed into stomacher bags, 10 ml of 10% oxalic acid (BDH) was added and the mixture homogenized in a stomacher for two minutes. In the case of other tissue samples, the oxalic acid was added in the same proportion (w/v) and treated likewise. One 1.0 ml aliquot of each homogenate was then diluted in 10-fold steps in Reid's salt base to $10^{-4}$; and from each dilution, 0.1 ml was inoculated onto the culture medium following the procedures described previously for sterile samples.

(b) Culture of faecal samples

One g of faeces was suspended and homogenized in 10 ml DW and allowed to settle for one hour at room temperature. Five ml of the supernatant (free from floating debris) was then transferred into 40 ml of 0.75% cetyl pyridinium chloride (CPC; Sigma). The mixture was held overnight at room temperature after which 0.1 ml aliquots of the sediment were inoculated onto paired dried slopes of the culture medium, with and without mycobactin, and incubated as described previously for sterile samples.

2.2.2 Identification

2.2.2.1 Microscopic examination of smears

Smears from suspect samples of faeces, intestines and lymph nodes or growth on culture media were prepared on alcohol cleaned microscope slides, air dried and then fixed by heating over a bunsen flame. The smears were stained by the ZN method and examined microscopically (x 1250).

The finding of clumps of small AFB either free or in cells or large numbers of single acid-fast rods of morphology consistent with that of M. ptbc was suggestive of the organism.

2.2.2.2 Growth requirements and cultural characteristics

Sub-cultures from primary isolates were grown on solid modified Dubos medium with and without mycobactin following the methods described previously in section 2.2.1.2. Slow growing (1-3 m.o. or more) greyish-white colonies of AFB appearing in the mycobactin-containing, but not in the non-mycobactin culture medium, were considered to be M. ptbc.

2.2.2.3 Pathogenicity for laboratory animals

Further proof for the identity of M. ptbc was obtained by inoculation of a culture suspension of the mycobactin-dependent AFB into rabbits, guinea pigs and chickens using the procedures

described in section 2.3.3.2. Cultures which were not pathogenic for these animals within 8-12 weeks after infection were regarded as being M. ptbc.

## 2.3 Infection of experimental animals

### 2.3.1 Preparation of the inoculum

The inoculum for infection was prepared following the method described by Brotherston and others (1961). Briefly, the growth of 8-10 weeks old culture of M. ptbc on solid Dubos medium was harvested into 10 ml of Watson-Reid salt base in a sterile stomacher bag and homogenized for two minutes. The suspension was transferred into a sterile universal bottle and allowed to stand at room temperature for at least 30 min for the large clumps to sediment. The supernatant was diluted in the salt base and standardized to Brown's opacity scale 2, equivalent to an estimated $10^8$ organisms/ml, and made up to the required volume.

### 2.3.2 Viable unit counts

A measurement of viable unit counts (VUC) of bacteria in the inoculum and tissues was made following the procedures previously described by Brotherston and others (1961). The inoculum of approximately $10^8$ organisms/ml was diluted in 10-fold steps in Reid's salt base to $10^{-5}$. Aliquots of 0.1 ml from each dilution were then inoculated onto paired dried solid modified Dubos medium slants and incubated accordingly. Tissues were treated as described previously under section 2.2.1.2.

For an inoculum, colony counts were made after 4-6 weeks of incubation, whereas for tissues, counts were made after eight and again after 12 weeks. The counts were made preferentially in slopes with 10 to 100 colonies. Counts for the inocula and each tissue were calculated by multiplying the mean of the numbers of colonies in replicate slopes inoculated with the same dilutions by the appropriate dilution factors.

2.3.3 Infection protocols

2.3.3.1 Deer and sheep

Deer and sheep were dosed orally by placing the inoculum onto the back of the pharynx by means of a short rubber tube attached to a syringe. The dose volume was five ml and contained approximately $10^8$ organisms/ml. Dosing was repeated weekly for 10 weeks. Viable unit counts in the doses given were checked by back titration of the first, middle and last doses following the procedures described previously in section 2.3.2.

2.3.3.2 Laboratory animals

Two young New Zealand white rabbits (4-6 m.o.), three Dunkin-Hartley guinea pigs (8-12 weeks) and two Rhode Island Red chickens (6-8 weeks) were used for testing one isolate of mycobacterium. The animals were given a single dose of 0.5 ml of the inoculum (section 2.3.1) administered parenterally. Rabbits were injected s/c into the neck fold, guinea pigs deep i/m into the inner thigh muscles, and chickens i/v into the wing-web vein.

## 2.4 Molecular characterization of M. paratuberculosis

Molecular characterization of isolates of M. ptbc from deer included sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of cell lysates, Western blotting, restriction endonuclease digestion of DNA and southern hybridization.

### 2.4.1 Analysis of cell proteins of M. paratuberculosis by SDS-PAGE and Western blotting

#### 2.4.1.1 SDS-PAGE

The electrophoretic separation of cell proteins in the lysates was carried out in a Protean, dual, vertical, slab gel cell (Bio-Rad Laboratories, Richmond, Calfornia) following the method of Laemmli (1970). A discontinuous buffer system was used.

(1) Preparation of the cell proteins

The cell proteins were prepared following the procedure described previously for the crude antigen for ELISA (section 2.1.1.4).

(2) SDS-PAGE procedure

The glass plates (170 x 140 mm) were assembled for 1.5 mm gels and resolving and stacking gels of the following compositions were used:

| Ingredient | Resolving gel | Stacking gel |
|---|---|---|
| Tris-HCl buffer (Sigma 7-9) | 375mM pH 8.8 | 125mM pH 6.8 |
| Sodium dodecyl sulphate (SDS; Sigma) | 0.1% (w/v) | 0.1% (w/v) |
| Acrylamide (Electran, BDH) | 10% (v/v) | 4% (v/v) |
| Sucrose (FSA) | 5% (w/v) | - |
| Ammonium per sulphate (APS; Sigma) | 0.05% (w/v) | 0.05% (w/v) |
| N',N',N'N-Tetramethylethylene-diamine (TEMED; Sigma) | 0.025% (v/v) | 0.025% (v/v) |

Before addition of freshly prepared APS and TEMED, the resolving monomer solution was degassed in a vacuum for at least 15 min. The gel was then cast by carefully pouring the solution in between the plates with a glass pipette to a level which allowed for one cm of stacking gel between the sample wells and the resolving gel. Care was taken to avoid trapping air bubbles. An overlay of stock stacking gel buffer was then immediately poured on the gel to prevent evaporation. After at least 45 min of polymerization, the overlay was poured off and the gel washed once with the stacking gel buffer. A stacking gel was cast after adding the APS and TEMED to degassed stacking gel monomer solution. Appropriate Teflon combs were then inserted, care being taken to avoid trapping air bubbles, and the gel was allowed to polymerize for at least 30 min. The combs were then carefully removed and the gel sandwich assembly released from the casting stand. After the sandwich assembly was attached and sealed to the top reservoir

tank, the wells were filled with SDS-PAGE electrode buffer which contained 192mM glycine (FSA), 25 mM Tris buffer and 0.1% SDS.

Each sample was diluted 1:1 (v/v) in a denaturing (sample) buffer composed of 62.5mM Tris-HCl buffer pH 6.8, 2% SDS, 10% (v/v) glycerol, 5% (v/v) 2-mercaptoethanol and 0.001% (w/v) Bromophenol blue (BDH). The mixture was then heated in a boiling waterbath for five minutes to completely dissociate the proteins.

Using a 50 µl Hamilton microlitre syringe (Aldrich Chemical Company Inc, Milwalkee, Wis, 53233, USA), the wells were loaded with the protein sample buffer mixture (50 µg crude protein). Standard markers of known molecular weight (MW), of the range 29000-205000 (MW-SDS- 200 kit; Sigma), were included on each gel.

After filling the bottom reservoir tank with two litres of the running buffer, the top reservoir was filled to the mark taking care not to disturb the samples. Air bubbles trapped at the anode end of the gel were removed with the bent end of a glass pipette. The cell was then connected to a power supply (Bio-Rad) and the polypeptides were separated at a constant current of 13 mA/gel. Each gel was run until the dye front was about one cm from the bottom of the plates (approx. 5-6h). After the electrophoresis was over, the apparatus was disassembled, the stacking gel sliced away and the resolving gel carefully removed from the glass plate for either staining or blotting.

(3) Staining of the gels

Gels were stained in duplicate, one with Coomassie blue and the other with silver stain.

(a) Coomassie blue staining

The gel was soaked in Coomassie blue stain which contained 0.04% Coomassie brilliant blue R250 (Sigma), 1% (v/v) trichloroacetic acid (BDH), 7.11% (v/v) glacial acetic acid (FSA) and 50% (v/v) ethanol (Hayman Limited Witham, Essex, England), for at least two hours and then destained in three complete changes of Coomassie blue destainer (7% (v/v) glacial acetic acid and 23% (v/v) ethanol in DW). Staining and destaining were carried out on an orbital shaker at room temperature.

(b) Silver staining

The method described by Morrissey (1981) was used for silver staining of the gels. All stages were performed on a shaker at room temperature and unless otherwise stated working volumes were 100 ml. The gel was pre-fixed in 50% (v/v) methanol (BDH) and 10% (v/v) glacial acetic acid for 30 min followed by 5% (v/v) methanol and 7% (v/v) glacial acetic acid for 30 min. The gel was then fixed in 10% (v/v) glutaraldehyde (BDH) for 30 min and rinsed in several changes of DW for 4-5 hours. After washing, the gel was soaked in 5 µg/ml dithiothreitol (DL-DTT; Cleland's Reagent; Sigma) for 30 min. The solution was then discarded and 0.1% (w/v)

silver nitrate (BDH) solution added. After equilibriation for 30 min, the gel was rinsed once in DW for 10 sec and then twice rapidly in 2 x 25 ml volumes of developer made from 50 µl 37% formaldehyde (BDH) in 100 ml 3% (w/v) sodium carbonate (BDH). This was followed by soaking the gel in 50 ml of fresh developer until stained to the desired level. Staining was stopped by adding five ml of 2.3M citric acid (BDH) directly to the developer and agitating for 10 min. The solution was then discarded and the gel washed several times in DW over a 30-minute period. To prevent bleaching during storage, the gel was soaked in 0.03% (w/v) sodium carbonate for 10 min.

Gels stained by either this method or the Coomassie blue stain were then photographed and dried in a gel drier (Bio-Rad model 483) at 60°C for at least two hours.

2.4.1.2 Western blotting

The antigenic characteristics of the SDS-PAGE separated fractions of the lysates were determined by Western blotting.

(1) Blotting procedure

The SDS-PAGE fractionated cell proteins were transferred electrophoretically from the gels onto transfer membranes using a modification of the method described by Towbin, Staehelin and Gordon (1979). The transfer was carried out in a Bio-Rad electroblot system. The blotting tank was filled with blot tank buffer (BTB) pH 8.3 which contained 25mM Tris buffer, 192mM

glycine and 20% (v/v) methanol. The blot pack was assembled and the scotch brite packs soaked in the BTB. Two pieces of Whatman 3MM paper cut to fit inside the closed frame were soaked in the tank buffer and then laid on top of the pad overlying the anode side of the frame. In this, and the subsequent stages, any air bubbles trapped between the layers were gently rolled out with a glass pipette. A transfer membrane (Immobilon P; Millipore Corporation, Bedford MA 01730) cut to slightly larger size than the gel was fixed in methanol for few seconds and then washed in DW. After the fixed membrane was soaked in tank buffer and carefully laid onto the wet blotting papers, the gel was then carefully laid on top of the wet membrane. The corners of the top of the gel and dye front were marked on the membrane in ink or pencil. After folding two pieces of wet blotting paper over the gel, the frame was closed, clamped and placed into the blot tank. The tank was then filled with tank buffer to the mark and the electrodes connected to the appropriate leads. The power supply was set to yield 300 mA and the elecrophoretic transfer allowed to continue for at least six hours.

(2) Probing

The blot pack was disassembled and the membrane removed from the frame and placed in a washbath containing blot wash buffer (BWB) pH 7.2. The BWB comprised a solution containing 0.5% (v/v) Tween 20, 0.35M sodium choride, 1mM ethylenediaminetetraaceticacid di-sodium salt (EDTA, dihydrate; Sigma) and PBS. Unless otherwise stated, all

washes were made in BWB with agitation at room temperature. The blot was washed three times for five minutes to remove the tank buffer and then stained by soaking and agitation in Ponceau S working solution (Sigma) for five minutes. The position of the standard protein size markers, and any important bands, were marked with ink or pencil. After removing the stain by rinsing quickly in DW and washing twice for five minutes in the wash buffer, the blot was blanked by incubating in 20 ml 50% (v/v) horse serum (diluted in BWB) for one hour at 37°C on a shaker. The blot was then washed repeatedly (3 short - 2 min, 3 long - 5 min, 3 short washes) and probed with the immune serum contained in a 20 ml mixture of a 1/40 dilution of the immune serum (0.5 ml), horse serum (3.0 ml) and wash buffer (16.5 ml). The reaction was allowed to continue on a shaker for one hour at 37°C. The blot was then washed repeatedly using a sequence of three short, three long and three short washes. Twenty ml of 1/200 dilution (in BWB) of the antispecies horseradish peroxidase (HRP) conjugated antibody was added and incubated at 37°C for one hour on a shaker.

(3) Developing

After repeated washing in the wash buffer, the blot was rinsed in HRP substrate buffer (0.1M Tris buffer, pH 7.4). This was followed by addition of the enzyme substrate which contained 5.0 mg diaminobenzidine (DAB; Sigma), 20 ml HRP substrate buffer and 60 µl 30% hydrogen peroxide. Agitation at room temperature was continued

until the desired level of staining was obtained. The reaction was stopped by rinsing the blot in tap water. The blot was then dried in air.

2.4.2. Analysis of genomic DNA of M. paratuberculosis by restriction endonuclease digestion and Southern hybridization

2.4.2.1 Preparation of genomic DNA

(1) Growth of the organisms

The organisms were grown following a modification of the method described by Whipple and others (1987). A one ml aliquot of a suspension of the organisms (approximately $10^8$ organisms) in Reid's liquid medium was inoculated into a 175 $cm^2$ polystyrene tissue culture flask containing 200 ml of liquid modified Dubos medium (with mycobactin J, 2 µg/ml). Two flasks were seeded for each isolate of mycobacterium. The flasks were placed in a horizontal position, so that the depth of the medium was <5 mm, and incubated static at 37°C until the turbidity (Å540) of the suspension was approximately 0.2. At that time, filter sterilized D-cycloserine (Sigma) was added to each flask to a final concentration of 1 mg/ml. After the contents were mixed well, cultures of M. ptbc were incubated for a further 18-24 hours, and those of M. avium (used for comparative studies), only for a further 3-4 hours. The organisms were harvested by centrifugation at 4000 rev/min (2600xg) for 30 minutes at 10°C. The cells were transferred into 1.5 ml eppendorf tubes and washed several times in Tris-EDTA-NaCl (TEN) buffer (50 mM Tris HCl pH 8.0, 100 mM EDTA, 150 mM NaCl) by

centrifugation (5000xg for 5 min) on a bench-top microcentrifuge (Minifuge GL, GMBH Osterode) to wash off the medium and as much as possible of the yellow tinge (from fungizone), from the cells. After the final wash, the cell pellets were stored at -20°C. Approximately 100-150 mg (wet weight) of cells were obtained from a flask of the culture.

(2) DNA isolation

To avoid possible shearing of DNA by disruptive methods of extraction such as those described by Baess (1974) and Hurley, Splitter and Welch (1987), DNA isolation from the mycobacterial cells was carried out, with slight modifications, following the enzymic lysis method described by Visuvanathan, Moss, Stanford, Hermon-Taylor and McFadden (1989). In short, approximately 100-150 mg (wet weight) of the cells were lysed following sequential treatment with 50 µl of subtilisin (100 mg/ml; Sigma) at 37°C for 18 hours, 100 µl of lysozyme (200mg/ml; Sigma) at 50°C for five hours. To complete the lysis, 50 µl of 20% (w/v) SDS and 50 µl of proteinase K (30 mg/ml; Sigma) were added, and the mixture incubated further for 20 hours, with addition of another 50 µl of proteinase K after 12-15 hours. Two phenol:chloroform:isoamyl alcohol (25:24:1; v/v) extractions were carried out and the DNA was spooled by ammonium acetate (1/5 of volume of 10M solution), ethanol (2 volumes of 100%) precipitation. After washing in 70% ethanol and brief desiccation, the DNA was resuspended in 100 µl of Tris-EDTA (TE) buffer (10 mM Tris HCl pH 8.0, 1 mM EDTA)

containing 10 µg/ml of boiled ribonuclease A (Sigma) and stored at -20°C, until use.

DNA yields were estimated by gel electrophoresis (section 2.4.2.3 (2)), by matching against known dilutions of a DNA standard run in parallel, on a mini-slab gel of 0.6% agarose (containing 0.5 µg/ml ethidium bromide) and visualized on an UV transilluminator (UVP, Cambridge UK). A yield of 75-200 µg of DNA was obtained from each extraction.

2.4.2.2 Genomic confirmation of M. paratuberculosis

M. ptbc was confirmed genomically by detection of a unique 279 bp DNA fragment (PCR 279) of the insertion element IS900, specific for M. ptbc (Green, Tizard, Moss, Thompson, Winterbourne, McFadden and Hermon-Taylor, 1989) amplified by the PCR.

(1) PCR amplification of the 279 bp DNA fragment specific for M. paratuberculosis

PCR amplification of the fragment was performed following the method described by Green and co-workers (1989) employing two oligonucleotide primers (P11 and P36). The amplification of the test DNA along with a plasmid DNA (pPN14; a gift from Dr. J. Hermon-Taylor) (positive standard) and DNA from a known M. avium organism (negative standard) were carried out in a 50 µl reaction mixture containing 67mM Tris-HCl pH 8.8, 16.6 mM ammonium sulphate, 6.7 mM magnesium chloride, 10 mM β-mercaptoethanol, 170 µg/ml bovine serum albumin (BSA), 0.5 mM of each of the four

deoxyribonucleotides (dATP, dCTP, dGTP, dTTP; Pharmacia), 500 µg each, of the primers 11 and 36 and 2.5 units of Taq DNA polymerase (Ampli Taq; ILS Ltd., London), made up to the required volume (50 µl), with deionized DW. The reaction mixes were overlaid with 50 µl volumes of mineral oil (Sigma) and subjected to 30 cycles of amplification in a DNA thermal cycler (Perkin-Elmer Cetus) under the following conditions: 60 seconds denaturation at 93°C, 60 seconds annealing at 58°C and 180 seconds polymerization at 72°C.

(2) Assay of the PCR-amplified DNAs

The mineral oil was aspirated out carefully, and 10 µl of each reaction mixture were assayed by electrophoresis, in parallel with molecular size markers (1 kb DNA ladder; Gibco), on a 0.6% agarose gel containing ethidium bromide (0.5 µg/ml) and then visualized on an UV transilluminator for the correct positioning of the 279 bp fragment.

2.4.2.3 Restriction endonuclease analysis

(1) RE digestion

Each DNA sample (3 µg) was digested with 20 units of the selected restriction endonuclease for 16-18 hours at 37°C under conditions specified by the manufacturer. Complete digestion was assayed by the inclusion of lambda DNA in the panel of DNAs digested by each of the enzymes used.

(2) Separation of the DNA restriction fragments

After adding Ficoll loading buffer (Appendix 1d) to a tenth of the final volume of the digest mixture, the DNA fragments were separated in parallel with a 1 kb DNA ladder on a 20 cm-long slab gel of 0.6% agarose by horizontal gel electrophoresis (BRL, Model H4). The gel was made from 2.4g agarose NA (Pharmacia, Uppsala, Sweden) in 400 ml Tris-borate-EDTA (TBE) buffer pH 8.3 (Appendix 1c) and ethidium bromide (0.5 µg/ml). The separation was carried out at a constant voltage of 35V for 16-18 hours. After the separation, the gel was placed on an UV transilluminator and photographed on high speed Polaroid 57 film. DNA restriction fragments of the organisms from each enzyme digest, were separated on one gel, for easy comparison.

2.4.2.4 Southern hybridization

The restriction fragments were blotted onto transfer membranes and hybridized to a radiolabelled DNA probe ($^{32}$P-PCR 279), by the method of Southern (1975), and the resulting patterns analysed.

(1) Transfer of the DNA restriction fragments from gels onto blots

The DNA fragments were transferred from gels onto blots following the procedures described by Sambrook, Fritsch and Maniatis (1989). The gel containing the restriction fragments was trimmed and then soaked in 500 ml of a denaturing buffer (1.5M

sodium choride, 0.5M sodium hydroxide) for 45 minutes with constant gentle agitation on a rotary platform. The gel was then rinsed briefly in deionized DW and soaked in 500 ml of a neutralizing buffer (1.0M Tris-HCl pH 8.0, 1.5M sodium chloride) again, with constant gentle agitation.

A piece of Whatman 3MM paper was wrapped around a stack of glass plates to form a support that is longer and wider than the gel. The wrapped support was placed inside a large baking dish. The dish was filled with transfer buffer (10 x SSC; Appendix 1e) until the level of the liquid reached almost to the top of the support. When the 3MM paper on top of the support was thoroughly wet, it was smoothed out of all air bubbles. A piece of nylon membrane (Hybond-N; Amersham U.K., Little Chalfont, United Kingdom) about one mm larger than the gel in both dimensions was cut and wetted in deionized DW and then immersed in transfer buffer for at least five min. The gel was removed from the neutralizing buffer and placed inverted on the support, centred on the wet 3MM paper. Any air bubbles between the two layers were rolled out. After the gel was surrounded with Parafilm, the wet membrane was laid carefully on top of the gel and any air bubbles smoothed out. Six pieces of 3MM paper cut to exactly the same size as the gel were wetted in 2 x SSC and placed on top of the wet membrane one at a time. Any air bubbles between the layers were rolled out. A stack of paper towels (5-8 cm high) cut to a size just smaller than the 3MM papers was placed on the 3MM papers. A glass plate was placed on top of the stack and weighed down with 500g weight. Transfer of the DNA

fragments from the gel onto the membrane was by capillarity and it was allowed to continue for 18-24 hours. The paper towels and the 3MM papers above the gel were removed and the membrane still attached to the gel was laid (gel side up) on a dry sheet of 3MM paper. After the positions of the gel slots were marked on the membrane with a ballpoint pen, the gel was peeled from the membrane and discarded. The membrane was soaked in 6 x SSC for five min at room temperature to remove pieces of agarose sticking to the membrane. After drying on a paper towel for at least 30 min, the membrane was sandwiched between two sheets of dry 3MM paper and baked for two hours at 80°C.

(2) Preparation of the $^{32}$P-PCR 279 DNA probe

(a) Isolation of the probe

The PCR 279 fragments from the plasmid DNA pPN14 and the PCR amplified DNAs of M. ptbc (section 2.4.2.2) were separated from the remaining reaction mixture (approx. 40 µl of each DNA tested) by gel electrophoresis, in a 0.6% low gelling temperature agarose gel containing 0.5 µg/ml ethidium bromide. The correct fragments were identified on a UV transilluminator and then excised, pooled together and purified using a Qiagen tip-20 (Hybaid Ltd., Middlesex).

(b) Radiolabelling of the probe

The probe was radiolabelled with $^{32}$P-$\alpha$dATP (Amersham Corp., Arlington Heights, 111), following the method described by Feinberg and Vogelstein (1984) using the solutions shown in Appendix 1f. The degree of incorporation of the radioisotope into the DNA probe was estimated by chromatographic separation of incorporated and isotope using a polygram strip (CamLab Ltd., Cambridge CB4 1TH) and 0.75M potassium phosphate pH 3.5 followed by autoradiography of the strip after drying. Incorporation was considered successful when it was more than 50%.

(3) Hybridization of the radiolabelled probe to the DNA restriction fragments

(b) Pre-hybridization of the blot

The blotted nylon membrane was pre-hybridized in 20 ml of a pre-hybridization buffer solution (Appendix 1g) in a sealed plastic bag incubated for 4-6 hours in a waterbath at 65$^{o}$C, with gentle rocking.

(b) Hybridization

Following denaturation by boiling for five minutes, the probe was added to 20 ml of a hybridization buffer solution (Appendix 1g) which was then pipetted into the plastic bag containing the pre-hybridized membrane. The bag was sealed and hybridization was allowed to proceed overnight in the waterbath at 65$^{o}$C, with gentle rocking.

(c) Washing of the membrane

After hybridization, the membrane was washed sequentially, at 65°C in 500 ml washes of 3 x SSC, 0.1% SDS (2 x 30 min washes), 1 x SSC, 0.1% SDS (2 x 30 min), and finally, in 0.1 x SSC, 0.1% SDS (2 x 30 min), to remove unbound probe.

(d) Autoradiography

The membrane was exposed to a Kodak X Omat film in a cassette lined with intensifying screens, for 16-18 hours at -70°C. The autoradiograph was then developed and analysed for the presence of restriction fragment length polymorphisms (RFLPs).

# CHAPTER 3

## Epidemiology and Pathology of Paratuberculosis in Red Deer

### INTRODUCTION

As indicated in the review of the literature in Chapter 1, knowledge of the prevalence of infection with M. ptbc in ruminants other than cattle is sparse. Likewise, reports on the pathology of the disease in deer are scanty and inconsistent.

Recent experience in the United Kingdom has shown considerable variation in the epidemiology and pathology of paratuberculosis in deer (McKelvey, 1987). The infection, especially in red deer has been found to affect a range of ages from yearlings to mature breeding stock and to be characterized clinically by sudden onset and rapid loss of condition and weight. In many, illness leads to diarrhoea and death with acute enteropathy and involution of mesenteric lymph nodes. For example, in 1985, in a herd of 400 breeding hinds on Farm 'A' - a source of material for the investigations described in this chapter - several yearlings developed these clinical and pathological features. From them and 13 clinically normal yearlings, acid-fast organisms which appeared similar to M. ptbc were identified. On the same farm in 1986, the disease affected five yearlings and a further 35 out of 400 hinds were found to have antibodies to M. ptbc. At slaughter, 41% of 59 yearlings and 73% of the seropositive hinds were found to have histological evidence of infection (McKelvey, 1987).

Thus not only does the clinical and post-mortem picture of paratuberculosis in deer seem to differ from that seen in cattle,

but also younger animals are affected and the incubation period and course of the disease appear to be shorter. Hence the investigations described in this chapter were intended (1) to obtain background epidemiological information on the prevalence of infection with M. ptbc in an affected herd of red deer, and in addition, to assess the potential value of vaccination in controlling infection; and (2) to describe the gross and microscopic pathology of the disease in naturally infected deer.

## RESULTS

### 3.1 Investigations on the prevalence of infection with M. paratuberculosis in an affected herd of red deer and the effect of vaccination on the infection in the herd

In a three-year study (1987 to 1990), the prevalence of infection with M. ptbc in an affected herd of red deer and the effect of vaccination on the infection in the herd were investigated by examination of mesenteric lymph nodes by histopathology and culture.

In the first year of the investigation, the nodes were obtained from a batch of 167 apparently normal 18-month to 2-year old red deer at slaughter. The animals came from farm A and had not been vaccinated against paratuberculosis. The ileocaecal and distal mesenteric nodes were collected from each animal at slaughter and processed for histopathology as described in section 2.1.2.2. A pool of the nodes from a group of 10 animals in the batch was also collected and processed for culture as described in section 2.2.1.2.

In the following year, 216 animals of the same age and origin, 201 of which were vaccinated against paratuberculosis within 24 hours of birth using a standard bovine vaccine (MAFF, CVL, Weybridge), were sampled for the lymph node investigation. Each sample was examined as above. During the third year, 47 vaccinated animals were sampled and examined in the same manner.

Table 3.1 summarizes the results of histopathology and culture for the three years. M. ptbc was isolated in culture from the node pool of the first year and the nodes of all animals with histological evidence of the organism in the other two years. Table 3.2 shows the relationship of the results of histopathology and culture for the second and third years. More positive animals were identified by the two tests combined than by either test alone.

The histopathological lesions in vaccinates were less severe and much less extensive than those of the unvaccinated deer. They were scanty with few or no organisms, and many were of tuberculoid nature, some of which were mineralized and segregated from the surrounding normal tissues. Figure 3.1 shows the proportion of animals with severe lesions.

During the study period, only five clinical cases of paratuberculosis were reported in the herd (in the 2nd yr). All were adult hinds.

## 3.2 Pathology

The pathology of paratuberculosis in deer was investigated by macro- and microscopic examinations of spontaneously infected red deer. Seven clinically affected adult hinds from farm A and

another unrelated deer farm (farm 'B') were transported to the Moredun Institute. All, except one hind which was received dead, were subjected to a number of ante-mortem examinations. These were clinical and faecal examinations, i/d tuberculin test and serology. After killing or death, the carcases were necropsied and selected tissues taken for histopathology and culture.

An eighth adult red deer hind from farm B with clinical manifestations of paratuberculosis was found to be infected with M. avium. This animal provided material for comparing the pathology of the two mycobacterial infections in deer.

### 3.2.1 Pathology of paratuberculosis in red deer

#### 3.2.1.1 Ante-mortem examinations

(1) Clinical findings

The six live paratuberculous hinds were checked for clinical abnormalities for a period ranging from 2 to 10 weeks. Symptoms were similar in all and included poor bodily condition and a dull, rough hair coat, with sometimes patches of alopecia (Fig. 3.2). The animals were generally bright, had good appetites and normal body temperatures. Their faeces were frequently soft and pasty, but two hinds were diarrhoeic. The hind quarters especially in the region of the perineum and hocks were frequently soiled by dried faecal material.

(2) Faecal examination

Fresh faecal samples were collected from the hinds soon after their arrival at the Institute, and thereafter, at two-week

intervals. The samples were subjected to microscopic examination of smears and culture for M. ptbc using the methods described previously in sections 2.2.2.1 and 2.2.1.2, respectively. Typical clumps of acid-fast organisms were detected in smears of four (Table 3.3) and M. ptbc was cultured from the same four samples.

(3) I/d tuberculin test and serology

The animals were tested for DTH within a day or two after arrival. The single i/d comparative tuberculin test was used following the method described in section 2.1.1.3. All six were negative to the test.

Blood for serology was collected from the animals soon after arrival and at killing and antibody to M. ptbc measured by ELISA following the method described previously under section 2.1.1.4. The results of serology are summarized in Table 3.3.

3.2.1.2 Post-mortem examinations

(1) Pathology

(a) Gross pathology

The animals were stunned with a captive bolt and exsanguinated. The carcases were examined for the presence of gross abnormalities and samples collected for histopathology and culture following the procedures described in section 2.1.2.1.

The general picture at necropsy was indicative of a chronic ill-thrift syndrome and the most consistent gross changes were emaciation and poor hair coat (Fig. 3.2). In addition, there was submandibular oedema, gelatinous atrophy of body-fat depots and serous effusion into body cavities, especially the peritoneum.

The lesions specific for paratuberculosis were confined to the intestines, mesentery and associated lymphatics. Changes in organs and tissues other than these were not apparent by gross inspection.

Non-paratuberculous pathological lesions were found in some of the deer (Nos. 26, 113 and 114). The findings included verminous pneumonia caused by Dictyocaulus viviparus and intestinal parasitism due to Oesophagostomum radiatum.

(i) Intestines

The intact intestines appeared grossly normal in some animals (Table 3.4), but in others, particularly in the region of the lower intestines, were slightly or moderately thickened and oedematous (Fig. 3.3). Changes in the mucosa were also variable (Table 3.4). In some, it appeared normal while in others, there were non-specific changes consisting mainly of patches of congestion and haemorrhages especially along crest folds. In others, the mucosa was irregularly or diffusely thickened and oedematous (Fig. 3.4), and in others in addition, it was haemorrhagic and focally eroded and ulcerated (Fig. 3.5). The specific intestinal changes were most extensive in the distal and middle small intestine with the haemorrhages, erosions and ulcerations being more pronounced in the middle small intestine. Changes in the caecum and colon were mild and the rectum and proximal small intestine were devoid of specific gross changes.

(ii) Mesentery and lymphatic channels

Changes in the mesentery were also variable (Table 3.4). In some, it was normal whereas in others it showed varying degrees of oedema and thickening. The grossly affected mesentery contained thickened, opaque afferent lymphatic channels interrupted along their course by miliary greyish-white nodules about 1-2 mm in diameter (Fig. 3.3).

(iii) Mesenteric lymph nodes

Mesenteric lymph nodes were either apparently normal (Table 3.4) or enlarged, oedematous and pale. The affected nodes were either soft or indurated. Those which were indurated frequently contained greyish-white foci (1-3 mm in diameter) that could be seen from the capsular surface and were gritty on cut-section.

(b) Histopathology

Specific microscopic changes were found mainly in the intestines, mesentery and associated lymphatics (Table 3.5) and they were dominated by varying degrees of infiltration and accumulation of epithelioid cells, macrophages and occasional multinucleated giant cells. In organs and tissues other than these, characteristic changes were seen only in the liver and retropharyngeal lymph nodes of some of the animals (Table 3.5).

(i) Intestines

In the intestines, specific changes were most extensive in the terminal and middle small intestine, less so in the caecum and proximal colon, and almost absent in the rectum and proximal duodenum. The cellular changes were mainly found in the mucosa and submucosa (Fig. 3.6), but where the mesentery was also involved, they extended into the serosa.

In the mucosa, the lamina propria was invaded by varying numbers of epithelioid cells and macrophages (Fig. 3.7). Giant cells were very occasionally observed. Other inflammatory cells which included lymphocytes, other mononuclear cells and granulocytic leucocytes, especially eosinophils, were also present but usually in inverse proportions to the larger invading cells. The cellular infiltrate was either diffusely distributed or in clusters and frequently more concentrated in the villous tips than towards the muscularis mucosa. Consequently, the cells caused considerable thickening and distortion of the tips and narrowing of intestinal crypts (Fig. 3.6).

In the submucosa, the specific cellular changes were found to be less extensive than in the mucosa (Fig. 3.6) but in some, especially in the most severely affected hinds, they were quite extensive causing marked thickening of this layer (Fig. 3.8). Focal microgranulomas containing aggregates of a few eosinophilic granulocytes and smaller mononuclear cells, and patches of necrosis were also found in those hinds. Peyer's patches were frequently enlarged and hyperplastic. They were invested or loosely

infiltrated by epithelioid cells and macrophages. In addition to some oedema, the remaining submucosal tissue was diffusely infiltrated by varying numbers of cells.

The sero-muscular layer was either normal or, in some animals, thickened and oedematous. Where involved, the serosa was found to be more cellular and to contain focal aggregations and loose diffuse infiltration of the invading cells extending from the mesentery. In some areas, these serosal changes extended into the muscular layer.

In ZN-stained sections of the affected tissues (Fig. 3.9), AFB were usually found in large numbers within epithelioid cells, macrophages and giant cells.

In the hinds with non-specific gross intestinal changes, the histologic picture was also non-specific. It was dominated by congestion, haemorrhages and a proportionally high number of lymphocytes and eosinophilic granulocytes in the mucosa and submucosa (Fig. 3.10). These changes were most extensive in the middle small intestine. In ZN-stained sections of the tissues there was no evidence of AFB.

(ii) Mesentery and lymphatic channels

Oedema and thickening were the most frequent histological changes in the affected mesentery. Specific changes consisted of some focal accumulations and diffuse infiltration of epithelioid macrophages, lymphocytes and other mononuclear cells.

The inflammatory changes found in the lymphatic channels were mainly parietal and focal. The walls of the channels were

thickened by focal aggregations of epithelioid macrophages and lymphocytes forming granulomatous plugs which projected either into the vessel lumen causing partial or complete obliteration of some channels or over the outer surface of the channels.

In ZN-stained sections of the mesentery, varying numbers of AFB were found within epithelioid macrophages.

(iii) Mesenteric lymph nodes

Specific microscopic changes in affected nodes consisted of varying degrees of diffuse infiltration and focal accummulations of epithelioid cells, macrophages and occasional giant cells, predominantly of the Langhans type. These granulomatous changes were limited mainly to the cortex (Fig. 3.11) and were predominantly paracortical. However in some nodes, supracapsular, subcapsular and follicular granulomatous changes were also present. In the severely affected nodes, the granulomas replaced much of the normal tissue, and changes associated with central necrosis, dystrophic mineralization and reactive fibrosis (Fig. 3.12) were frequently found in sections made from the nodes with focal gritty lesions. Apart from oedema, the medulla was almost devoid of specific changes.

In ZN-stained sections made from affected nodes (Fig. 3.13), AFB were usually found in large numbers within epithelioid cells, macrophages and the necrotic masses.

Non-specific histological changes found even in the non-grossly affected nodes included occasional clusters of histiocytic cells often laden with yellowish-brown material and marked depletion of

lymphoid cells in several lymphatic nodules. The germinal centres of such nodules were sometimes nearly empty or partially filled with homogenous or onion-sheath like eosinophilic deposits (Fig. 3.14).

(iv) Other organs and tissues

Liver

Focal microgranulomas (Fig. 3.15) containing lymphoid cells and a few epithelioid cells and macrophages were the characteristic changes found in affected livers.

Retropharyngeal lymph nodes

Specific histologic changes in the retropharyngeal nodes were similar to those found in the mesenteric nodes, but they were mild and without necrosis, mineralization and fibrosis.

(c) Culture of tissues for M. paratuberculosis

The various tissues taken for culture at autopsy were processed following the procedures described under section 2.2.1.2. M. ptbc was isolated only from the mesenteric nodes and the intestinal mucosa (Table 3.6).

3.2.2 Pathology of avian tuberculosis in red deer

The pathological features of the hind infected with M. avium were investigated following the methods used in the previous study (section 3.2.1).

3.2.2.1 Ante-mortem examinations

As the animal was severely affected, it was kept alive for less than 24 hours after arrival at the Institute. During this period, it was examined for clinical abnormalities and sampled for faecal examination and serology.

(1) Clinical findings

The main clinical signs, as presented in Figure 3.16 were indistinguishable from those described previously under section 3.2.1.1 for the paratuberculous hinds but were more severe.

(2) Faecal examination

AFB were found in faecal smears and a slow-growing mycobactin-independent AFB was isolated in culture.

(3) Serology

By ELISA (section 2.1.1.4) using the M. ptbc antigen, the hind was found to be weakly positive (OD = 0.05).

3.2.2.1 Post-mortem examinations

(1) Pathology

(a) Gross pathology

The general post-mortem picture of the carcase was similar to that of the hinds with paratuberculosis. However in this animal, the gross lesions were much more severe and extensive. Apart from

the intestines, mesentery and mesenteric lymphatics, gross lesions were also found in the tonsils and retropharyngeal and mediastinal lymph nodes.

(i) Intestines

Gross intestinal lesions were most extensive in the terminal and middle small intestine diminishing towards the proximal small intestine and the rectum. The serosal surface of the small intestine was markedly thickened and traversed by numerous, firm, corded, tortuous, greyish-yellow lymphatic channels leading into the mesentery (Fig. 3.17).

The intestines showed marked thickening of the entire wall. The mucosa especially of the distal and middle small intestine was irregularly thickened, roughened and oedematous. Extensive haemorrhages and focal erosions and ulcerations were also present, especially in the region of the middle small intestine (Fig. 3.18). Changes in the caecum and proximal colon were similar but less severe than those found in the small intestine.

(ii) Mesentery and lymphatic channels

Macroscopic changes in the mesentery were extensive and severe. The tissue was markedly thickened into a completely opaque solid greyish-yellow mass (Fig. 3.19) containing numerous corded lymphatic channels extending from the serosal surface of the bowel to the mesenteric lymph nodes. Interrupting the thickened channels along their course were miliary greyish-yellow nodules measuring 2-5 mm in diameter which gave the vessels a beaded appearance.

(iii) Mesenteric lymph nodes

The mesenteric lymph nodes were markedly enlarged measuring up to 4-5 cm in diameter, indurated and almost completely joined up to form a long greyish-white cord (Fig. 3.19). On cut-section, gritty greyish-white foci were found.

(iv) Other organs and tissues

Specific gross pathological changes in organs and tissues other than the intestines, mesentery and associated lymphatics were found only in the tonsils and the retropharyngeal and mediastinal lymph nodes. These were moderately enlarged and oedematous and in addition, they contained miliary greyish-yellow nodules about 1-2 mm in diameter visible from their outer surfaces. On cut-section, the nodules appeared as hard greyish-white foci.

(b) Histopathology

The specific microscopic changes were basically similar to those found in the hinds infected with M. ptbc but like the gross lesions, they were severe and extensive. In addition, they were disseminated into organs and tissues other than the ones grossly affected.

(i) Intestines

The intestinal mucosa, especially in the villous tips (Fig. 3.20), was heavily infiltrated by epithelioid cells, macrophages and occasional giant cells, causing considerable thickening of the tips.

The submucosa was also thickened. It contained large necrotic granulomas surrounded by fibrous tissue. In the granulomas, necrotic foci containing nuclear fragments of degenerated cells surrounded by epithelioid cells, macrophages, occasional giant cells and fibrous tissue were the most frequent features. In Peyer's patches, lymphatic follicles were depleted of lymphoid cells, and were surrounded and infiltrated by epithelioid cells and macrophages (Fig 3.21). The vicinity of the patches was heavily infiltrated by large phagocytic cells. Much of the remaining submucosa was oedematous and fibrotic.

The sero-muscular layer was thickened and oedematous. The serosa in particular was markedly thickened and fibrotic and it contained necrotic granulomas similar and larger than those found in the submucosa.

The intestinal changes in general were most severe and extensive in the middle and lower small intestine and less so in the caecum and colon. In ZN-stained sections of the intestines, numerous AFB were seen within epithelioid cells, macrophages and the necrotic masses (Fig. 3.22).

(ii) Mesentery and mesenteric lymphatics

Changes in the mesentery were extensive and severe. Numerous granulomas with large foci of necrosis some of which were mineralized were present and most of the remaining tissue was fibrotic. Blood vessel walls were markedly thickened (Fig. 3.23) and lymphatic channels showed focal endo- and peri- lymphangitis.

(iii) Mesenteric lymph nodes

Changes in the mesenteric lymph nodes were also severe and extensive. They were dominated by massive necrosis, mineralization and reactive fibrosis. The remaining tissue was infiltrated by varying numbers of epithelioid cells and macrophages. Giant cells were occasionally seen. In ZN-stained sections, numerous AFB were seen within many of the large phagocytic cells and necrotic masses.

(iv) Other organs and tissues

Specific lesions in other organs and tissues were found in the tonsils, retropharyngeal, mediastinal, prescapular and prefemoral lymph nodes, liver, spleen, kidneys and lungs.

Tonsils

The loose lymphatic tissue in the tonsils was heavily infiltrated by epithelioid cells and macrophages (Fig. 3.24). Discrete necrotic granulomas were also present.

Retropharyngeal and other carcase nodes

The specific lesions in the retropharyngeal lymph nodes were similar in extent and severity to those of the mesenteric chain. However changes in the mediastinal, prescapular and prefemoral lymph nodes were also similar but scanty and without necrosis, mineralization and fibrosis and they were also usually associated with oedema of the medulla.

Liver

Scattered, discrete, microgranulomas containing epithelioid cells, macrophages, lymphocytes and Küpffer cells were present in the liver. These lesions though similar were more numerous than those found in the paratuberculous hinds.

Spleen

Islets of epithelioid cells and macrophages, some with giant cells, in the splenic corpuscles were the common microscopic lesions found in the spleen (Fig. 3.25).

Kidneys

In the kidneys, lesions were frequently found in the glomerular tufts (Fig. 3.26) and less so in the interstitium. The tufts were thickened due to infiltration by epithelioid cells, macrophages and lymphocytes.

Lungs

Scattered focal thickening of the interalveolar walls (Fig. 3.27) due to infiltration of the septa by epithelioid cells, macrophages and some lymphocytes, was the prominent histopathological picture presented in the lungs.

In ZN-stained sections of these organs and tissues, AFB were usually seen in small numbers within epithelioid cells and macrophages.

(c) Culture of tissues

A slow growing mycobactin-independent AFB was isolated in culture from the intestinal mucosa, mesenteric lymph nodes, tonsils, retropharyngeal and mediastinal lymph nodes. The organism was tested for pathogenicity for rabbits, guinea pigs and chickens following the procedures described in section 2.3.3.2. It was found to be pathogenic only for the chickens, where tuberculous granulomas were found mainly in the spleen, liver and lungs (Fig. 3.28), thus qualifying for the identity of M. avium.

## DISCUSSION

### Prevalence of infection in the herd

The rate of infection of up to 38.3% (Table 3.1) encountered in the herd in the first year of the investigation among what appeared to be apparently normal young animals, suggests a high prevalence of infection with M. ptbc in deer. The figure is likely to have been even higher had all the animals been subjected to cultural examination of the nodes, a tendency observed in the other two years (2nd and 3rd) where the figure for the combined results (Table 3.2) was higher than the separate figures from either histopathology or culture (Table 3.1). This high rate of infection in deer was further supported by the results of a separate investigation - not included in this thesis - of mesenteric lymph nodes from 12 randomly selected apparently normal red deer of similar age originating from farm B which revealed 7 (58%) histopositive animals. This high rate of infection suggests a high susceptibility of the deer to infection with M. ptbc.

The rate of infection recorded in this investigation is higher than that reported for cattle in clinically normal herds (7-18%) in Great Britain (Taylor, 1949; Rankin, 1954; Smith, 1954b) and in the United States (Arnoldi and Hurley, 1983; Chiodini and Van Kruiningen, 1983b), but falls within the range (as much as 50%) reported in chronically affected herds (Withers, 1959; Sherman, 1985).

Although clinical paratuberculosis has been reported in this herd of deer and among fawns (Bourgeois, 1940; Libke and Walton, 1975) and older deer (Vance, 1961; Buxton, 1987), the outcome of allowing the animals described in this investigation to live longer, especially the positives, was not ascertained. No attempts were made to test the animals for faecal excretion of organisms and intestinal pathology. However it is evident that a good proportion of the histopositives (Table 3.1) had microscopic evidence of organisms, and as observed in years 2 and 3, M. ptbc was isolated from nodes of all animals with histological evidence of the organism. Therefore, as with other ruminants (Julian, 1975), it is likely that under conditions of stress and other enhancing factors, such asymptomatic carriers will become sick and/or active shedders of organisms forming a potential source of infection for herdmates and other susceptible animals in the shared environment. However the possibility of complete recovery in some of the animals as previously reported in other ruminants (Gilmour et al., 1978) cannot be ruled out. The failure to find or isolate organisms in a number of the histopositives (Table 3.2) supports this possibility.

Effect of vaccination on infection in the herd

The prevalence determined from the histopathological examinations of the vaccinated group in the second year was 15% and contrasted with the 38% encountered in the non-immunized group of the first year. The difference was very highly significant ($p << 0.001$) (Table 3.1). There was also a considerable reduction in the severity of infection as determined by the smaller proportion of histopositives with severe lesions (Fig. 3.1) and microscopic evidence of organisms (Table 3.1). Furthermore it is worthy of note that within the three-year study period, only five clinical cases of paratuberculosis were recorded in the herd, and these were among adult hinds born before the vaccination programme was instituted. However, as determined by the combined results of histopathology and culture, the vaccinates of the third year appeared to have a higher rate of infection (46.8% vs 20.4%) and a higher proportion (9/22 vs 10/41) of animals which were culture positive but histonegative than those of the second year. Nevertheless the difference was not significant (Chi-square = 2.002, $p > 0.05$).

Thus the findings in this investigation as in previous observations in sheep and cattle (Spears, 1959; Nisbet et al., 1962; Stuart, 1965a) suggest that vaccination in this herd of deer did not eliminate the infection, but only reduced the rate and severity of infection and the frequency of clinical cases of the disease.

Pathology

The main clinical signs of paratuberculosis in the deer were poor bodily condition and emaciation. Diarrhoea was present only in some animals. These clinical features of the disease in the hinds are similar to those described for paratuberculosis in cattle, sheep and goats (Blood et al., 1983).

Like previous isolated reports of paratuberculosis in red deer (Bourgeois, 1940; Vance, 1961; Jørgensen and Jørgensen, 1987) and other species of deer (Hillermark, 1966; Libke and Walton, 1975; Chiodini and Van Kruiningen, 1983a; Buxton, 1987), the pathological lesions manifested were not consistent. Of particular interest were lesions found in the intestines and associated lymph nodes. Although all the seven paratuberculous animals were severely affected clinically, some did not show the characteristic inflammatory response in the intestines and mesenteric nodes associated with M. ptbc infection in other ruminants and there was no microscopic evidence of organisms. Furthermore they neither had a DTH nor specific antibodies to M. ptbc and it was only through isolation of the organism from the mesenteric lymph nodes that the disease was confirmed. In another category of animals, the intestines were apparently normal macroscopically but severely affected histologically. The lack of correlation between the degree of clinical signs and the magnitude of the lesions is not specific for the paratuberculous deer described in this investigation. It is also known to occur in paratuberculous cattle (Hallman and Witter, 1933), sheep (Stamp and Watt, 1954) and goats

(Levi, 1948; Harding, 1957). Similar observations were reported in previous cases of paratuberculosis in red deer (Vance, 1961) and other species of deer (Libke and Walton, 1975; Chiodini and Van Kruiningen, 1983a). Hence in the absence of histopathology and the culture of organisms from the tissues, such cases are likely to be missed in routine immunological and post-mortem examinations.

However in some animals, obvious gross and histological intestinal and node lesions were present. In these animals, apart from irregular thickening of the intestinal mucosa, the characteristic corrugation of the mucosa common in bovine paratuberculosis (Jubb et al., 1985) was not present. Focal necrosis in the submucosa and necrosis, mineralization of some of the necrotic foci and reactive fibrosis in the mesenteric lymph nodes, changes frequently associated with paratuberculosis in sheep (Howarth, 1932; Stamp and Watt, 1954; Nisbet et al., 1962) and goats (Levi, 1948; Majeed, 1972), were present in some of the hinds with severe lesions. However, the degenerative changes in the submucosa were less common than those in the nodes. Similar lesions in the mesenteric nodes have been reported previously in red deer spontaneously infected with M. ptbc (Jørgensen and Jørgensen, 1987). Thus the lesions of paratuberculosis encountered in this study support the previous observations and suggest that the pathognomonic gross and histological lesions of paratuberculosis in deer closely resemble those of the disease in sheep and goats more than in cattle.

The clinical and pathological findings in the single case of avian tuberculosis in deer were similar to those of the

paratuberculosis cases. However the lesions in avian tuberculosis were more severe and widely distributed into organs other than the intestines and associated lymphatics. The close resemblance is bound to cause confusion in the differential diagnosis of the two infections in deer. Previous reports of avian tuberculosis in deer (Hopkinson and McDiarmid, 1964; Blaxter, Kay, Sharman, Cunningham and Hamilton, 1974) describe a generalized form of the disease which can cause further confusion with tuberculosis. However tuberculosis in red deer (Witte, 1940; Stuart, et al., 1988) and other species of deer (Belli, 1962; Towar, Scott and Goyings, 1965; Sawa, Thoen and Nagao, 1974; Jones, Manton and Cavanagh, 1976; Wilson and Harrington, 1976; Robinson, Phillips, Stevens and Storm, 1989) has often been associated with the formation of abscesses of varying forms and sizes, in various lymph nodes and organs.

Nevertheless despite differences among the mycobacterial infections, avian tuberculosis and infections with other atypical mycobacteria (Jones et al., 1976; Jørgensen and Clausen, 1976; Matthews et al., 1981) are of concern in the detection and control of tuberculosis and paratuberculosis in deer.

# CHAPTER 4

# Pathogeneses of Paratuberculosis and Avian Tuberculosis in Red Deer and Sheep

## INTRODUCTION

The epidemiological observations of paratuberculosis described in Chapter 3, indicated a high susceptibility of deer to infection with M. ptbc. Furthermore, paratuberculosis and avian tuberculosis in naturally infected deer are described as being clinically and pathologically similar.

Apart from the few field reports of paratuberculosis and avian tuberculosis in deer, very limited experimental infections with M. ptbc or M. avium have ever been conducted in these animals (Orr, Hunter, Brand and Owen, 1978; Williams et al., 1983). For a better understanding of the reaction of deer to the two mycobacteria, the pathogeneses of paratuberculosis and avian tuberculosis were investigated by experimental infection of very young red deer calves using isolates of M. ptbc and M. avium from naturally infected, clinically affected red deer. The experiment on the pathogeneses of the two infections was also repeated in lambs to assess the relative susceptibility of deer to M. ptbc and M. avium.

## RESULTS

### 4.1. Pathogeneses of paratuberculosis and avian tuberculosis in red deer

Fifteen 10-day old red deer calves obtained from a herd in which tuberculosis and paratuberculosis had never been diagnosed were

used for the investigation. They were placed into three separate groups of five calves. One group was challenged with M. ptbc, another with M. avium and the third was used as an uninfected control group and was given sterile Reid's liquid medium. The methods used for preparation of the inocula and the protocol for infection have been described in Chapter 2. To prevent cross-infection, the groups were maintained in separate houses in an isolation unit and separate clothing and equipment was used for handling and dosing each group.

During the course of the experiment, all animals were checked regularly for development of clinical abnormalities. They were also tested for faecal excretion of organisms and serology at day 0 and at 4-week intervals and for DTH during week 8 and at 8-10 week intervals threafter. The calves challenged with M. avium were not tested for DTH because they were killed before the scheduled time for testing. After killing, all carcases were subjected to gross, histopathological and cultural examinations. The methods used for the various examinations have been described in detail in Chapter 2.

Within the first two weeks of the experimental period, two of the calves challenged with M. avium and one of the control animals died from causes other than paratuberculosis or avian tuberculosis. Of the M. ptbc infected group, one of the calves was killed during week 15, another during week 36 and the rest during week 48, together with two from the control group. The remaining three M. avium infected calves had to be killed before the end of the dosing period because of their poor clinical condition. One was killed

during week 6 and the others during week 8. The procedure used for killing was as described in section 3.2.1.2. Table 4.1 summarizes results of the various examinations performed on the animals.

### 4.1.1 Clinical signs

Clinical signs in both infected groups were similar and of sudden onset. The main signs included rapid loss of condition, dull, rough, hair coat and unformed, pasty, faeces (Fig. 4.1 and 4.2). The clinical course of infection in both groups ran for a period of 1-3 weeks, ending up in death or recovery. The four control animals were apparently normal after the second week of the experiment.

In the group dosed with M. ptbc, clinical signs were apparent in three by week 15. One of them became more severely affected and had to be killed during the same week. The others appeared to have recovered clinically by week 19. However during week 35, one of them (No. 166) became sick again and was killed during week 36.

All the three M. avium dosed calves became severely affected clinically during weeks 6-8.

### 4.1.2 Faecal excretion of organisms

In both infected groups, organisms were detected in faeces only in the clinically affected animals during the period when signs of disease were apparent. However no organisms were detected in faeces of deer No. 166 during the relapse period. Organisms were not found in control animals.

4.1.3 Delayed-type hypersensitivity

Four of the calves dosed with M. ptbc and one of the control animals were found to be positive during week 8, but by week 19 and thereafter, they had little or no measurable skin reactivity (Table 4.2). One of the M. ptbc dosed DTH-positive calves showed a higher reactivity to the bovine PPD than the avian PPD. The reactor from the control group showed higher sensitivity to the avian PPD.

4.1.4 Serology

In calves dosed with M. ptbc, antibody to the bacillus was detected in three from weeks 12 to 19 (Fig. 4.3) whereas those dosed with M. avium and the control animals were all seronegative.

4.1.5 Pathology

4.1.5.1 Calves dosed with M. paratuberculosis

(1) Gross pathology

The carcases of the two animals killed during weeks 15 and 36 were emaciated. They had serous effusion into their abdominal cavities. In addition, the latter had atrophic body-fat depots. Specific macroscopic lesions were found in the intestines and mesenteric lymph nodes. Node lesions were found in all the animals but lesions in the intestines were apparent only in three (Table 4.3). While severe in No. 107, the intestinal lesions were less obvious in the others.

(i) Intestines

In general, lesions in the intestines were most severe in the terminal and middle small intestine and less so in the caecum and colon. The rectum and proximal small intestine were apparently normal. In the most severely affected calf, the serosal surface was thickened and oedematous. Peyer's patches were prominently enlarged. The mucosa was irregularly thickened, reddened and focally eroded. Frequent cauliflower-like ulcers were found in the mucosa in the region of the Peyer's patches. Slight thickening of the mucosa at the distal end of the small intestine and the ileocaecal valve in one (No. 166) and a firm discrete nodule measuring 2-3 mm in diameter in the middle small intestine of the other (No. 78), were the significant lesions found in the two less obviously affected animals.

(ii) Mesentery and lymphatic channels

In only the most severely affected calf were the mesentery and afferent lymphatic channels grossly affected. The mesentery was thickened and oedematous and it contained thickened, beaded, opaque, lymphatic channels.

(iii) Mesenteric lymph nodes

Gross lesions were found in the mesenteric lymph nodes of all calves. However they were most severe in calf No. 107. In that animal, the nodes were markedly enlarged measuring 4-5 cm in diameter, pale, firm and oedematous. They frequently contained

greyish-white foci that were gritty on cut-section. Lesions in the nodes of the other animals were less severe and more obvious in the distal than middle and proximal mesenteric nodes.

(iv) Other organs

Macroscopic lesions in organs other than the intestines and mesenteric lymphatics were non-specific and were found only in the severely affected calf. The lesions included slight to moderate enlargement, congestion and oedema of the tonsils, some carcase nodes and parenchymatous organs.

(2) Histopathology

Specific microscopic lesions were mostly found in the intestines and mesenteric lymphatics (Table 4.4), but in the severely affected calf, lesions were also apparent in the tonsils, some other lymph nodes, the liver, spleen, kidneys and lungs.

(i) Intestines

Microscopic lesions in the intestines were found only in the three animals with apparent gross lesions. In the severely affected calf, they were found in the small intestine, caecum and colon, being more severe and extensive in the distal and middle small intestine. However in the other two, the lesions were mild and localized, and most were found in close association with lymphatic nodules.

In the severely affected calf (No. 107) the mucosa was focally eroded and congested. The lamina propria especially of the villous tips was heavily infiltrated by epithelioid cells and macrophages.

Consequently, the tips became thickened considerably obliterating many of the intestinal crypts. However the entire mucosa was not obviously thickened. Focal aggregations and diffuse infiltration by lymphocytes, macrophages, epithelioid cells and granulocytic leucocytes, predominantly eosinophils, were found in some parts of the lamina propria. At some points, especially in the region of Peyer's patches, the mucosa was deeply ulcerated leaving the submucosa exposed (Fig. 4.4). In the submucosa, prominent changes were found in and around the Peyer's patches. The submucosa in this region was markedly thickened. The lymphatic follicles were enlarged and hyperplastic and some of them were surrounded and loosely infiltrated by epithelioid cells and macrophages. In severe cases, the cells replaced most of the normal tissue reducing the follicles to mere focal aggregates of a small number of lymphoid cells. Varying numbers of epithelioid cells and macrophages were frequently found concentrated in the interfollicular zones, especially in areas close to the muscularis mucosa. Giant cells were rarely seen. Other submucosal lesions included foci of necrosis infiltrated heavily by eosinophilic granulocytes, and surrounded by some macrophages, a few epithelioid cells and other mononuclear cells. Some of the foci were confluent, and had evidence of mineralization. In the rest of the submucosa were oedema, congestion and invasion by the various inflammatory cells and the development of granulation tissue. The serosa was irregularly thickened and oedematous. It was more cellular than normal, being loosely infiltrated by macrophages, lymphocytes and other mononuclear cells.

In the two less obviously affected animals, lesions were few and localized and were found only in the ileocaecal valve in No. 166 and the nodular swelling in No. 78. In the ileocaecal valve, the submucosa was thickened and lymphatic follicles were enlarged and hyperplastic. A large discrete non-capsulated granuloma (Fig. 4.6) containing epithelioid cells, macrophages and a few giant cells was found in the submucosa of the valve. A similar granuloma was found at the other site.

In ZN-stained sections of the affected intestines, AFB were seen in large numbers within epithelioid cells and macrophages in the severely affected animal. No organisms were found in the others.

(ii) Mesentery and lymphatic channels

The mesentery was thickened, oedematous and more cellular than normal (Fig. 4.5). Walls of afferent lymphatic channels and blood vessels were thickened. Those of blood vessels were thickened symmetrically as a consequence of hyperplasia of muscle cells of the tunica media whereas in the lymphatic channels, the walls were frequently asymmetrically thickened by focal accumulations of macrophages, lymphocytes and other mononuclear cells.

(iii) Mesenteric lymph nodes

The node capsule was either normal or thickened by various inflammatory cells and granulation tissue. There was little cortico-medullary distinction. Most of the normal lymphoid tissue of the cortex was replaced by extensive foci of necrosis. The necrotic masses contained eosinophilic granulocytes and remnants of the nuclei of dead cells. Foci of central mineralization were

found in some of the necrotic masses (Fig. 4.7). Epithelioid cells, macrophages, occasional giant cells and loose fibrous connective tissue enclosed the necrotic areas and replaced most of the remaining normal cortical tissue. The medulla was devoid of the specific changes. In ZN-stained sections of the nodes, AFB were found within epithelioid cells, macrophages, giant cells and necrotic masses. Organisms were numerous only in the severely affected calf.

(iv) Other organs

Histological lesions in organs other than the intestines and mesenteric lymphatics were found in the tonsils, some carcase nodes, liver, spleen, kidneys and lungs of only the severely affected calf. The common lesions found in the tonsils were focal aggregations of epithelioid cells and macrophages in the loose lymphoid tissue. In lymph nodes other than the mesenteric nodes, lesions were found in the retropharyngeal, bronchial, mediastinal, prescapular and prefemoral nodes. The lesions were similar to those found in the mesenteric nodes but less severe. However those in the retropharyngeal and bronchial nodes were equally severe and extensive. Lesions in the liver, spleen, kidneys and lungs were similar to those described for the hind affected by avian tuberculosis (Chapter 3).

In most of the organs, congestion, some haemorrhages and oedema were the accompanying non-specific inflammatory changes. In ZN-stained sections of the affected organs, AFB were found in numbers within epithelioid cells and macrophages.

4.1.5.2 Calves dosed with M. avium

(1) Gross pathology

The carcases were emaciated and they contained serous effusion into their abdominal cavities. In general, the specific gross lesions in all the three calves dosed with M. avium were basically similar to those described for the severely affected M. ptbc infected calf (Table 4.3). However lesions in the calf killed during week 6 were less extensive. In that animal, the main macroscopic lesions were enlargement of Peyer's patches, congestion and irregular thickening of the mucosa of the distal small intestine and moderate enlargement and oedema of the mesenteric lymph nodes. In the other calves, the intestinal lesions were more extensive, and in addition, they were accompanied by haemorrhage, erosion and ulceration of the mucosa (Fig. 4.8).

(2) Histopathology

Specific microscopic lesions were found in the intestines and mesenteric lymph nodes, but in the two calves with more extensive gross lesions, histological lesions were also found in other organs (Table 4.4).

(i) Intestines

Microscopic lesions in the intestines were also basically similar to those described for the severely affected M. ptbc infected calf and were also most extensive in the lower and middle small intestine, and less so, in the caecum, colon and proximal small intestine. However in calf No. 124, the lesions were less

extensive and less severe and were limited to the lower small intestine. They were most obvious in and around the Peyer's patches. The lymphatic follicles were enlarged and hyperplastic. Small foci of a few epithelioid cells and macrophages were seen in the interfollicular zones immediately below the muscularis mucosa and in the lamina propria of the villous tips of the overlying mucosa. In those sections, organisms were more numerous in the villous tips than in the other parts. Some of the lymphatic follicles were surrounded by epithelioid cells and macrophages and others were focally or loosely infiltrated by the cells. In sections with more advanced changes, the lesions were more confluent and the cellular changes more extensive, with the invading cells replacing many of the lymphatic follicles. In the calves with more severe lesions, the mucosa was eroded, congested, focally ulcerated and markedly thinned (Fig. 4.9). Focal microgranulomas containing granulocytic leucocytes - predominantly eosinophilic, some macrophages, epithelioid cells and other mononuclear cells were also found in the mucosa (Fig. 4.10). The submucosa was markedly thickened. Lymphatic follicles were enlarged, hyperplastic and infiltrated heavily by the inflammatory cells (Fig. 4.9). Also in the submucosa, were extensive necrotic granulomas heavily infiltrated by the granulocytic leucocytes and other cells. They were commonly located in areas immediately below the muscularis mucosa. The serosa was thickened and loosely infiltrated by the inflammatory cells (Fig. 4.11). Afferent lymphatic channels were focally thickened to cause near or complete obliteration of some of them (Fig. 4.11 and 4.12).

In ZN-stained sections of the intestines, AFB were seen in large numbers within epithelioid cells, macrophages and necrotic masses. The organisms were most numerous in calf No. 121.

(ii) Mesentery and lymphatic channels

Pathological changes in the mesentery and afferent lymphatics were found in the two calves with more extensive lesions and were similar to those found in the calf infected with M. ptbc.

(iii) Mesenteric lymph nodes

Mesenteric node lesions were also indistinguishable from those found in the calves infected with M. ptbc. However in the calf with less extensive lesions, the histopathological changes were not accompanied by necrosis, mineralization and extensive fibrosis. In all, congestion and some haemorrhages, especially in the corticomedullary region, were other changes. In ZN-stained sections of the nodes, AFB were found in large numbers within epithelioid cells, macrophages, giant cells and the necrotic masses.

(iv) Other organs

The histological lesions in organs other than the intestines and their draining lymphatics were similar to those described for the M. ptbc infected calf and were found in the tonsils, retropharyngeal, bronchial, mediastinal, prescapular and prefemoral lymph nodes, liver, spleen, kidneys and lungs. Lesions in the spleen and nodes other than the retropharyngeal and bronchial lymph nodes were not found in the calf with less extensive lesions. In

ZN-stained sections of the organs, AFB were found in all organs only from one of the calves. In the others, organisms were found only in the tonsils, retropharyngeal and bronchial lymph nodes.

4.1.5.3 Control animals

In the DTH-positive control animal, gross and microscopic lesions were found only in the distal mesenteric lymph nodes. The changes were moderate and indistinguishable from those described for the calves infected with M. ptbc. In ZN-stained sections of the nodes, AFB were few and were found within epithelioid cells, macrophages and necrotic masses.

4.1.6 Culture of tissues

In the group dosed with M. ptbc, mycobactin-dependent AFB were recovered from the mesenteric lymph nodes of all, and from the intestinal mucosa, tonsils and the other lymph nodes of only the calf with severe lesions. However the organism was recovered in large numbers only from the severely affected calf.

In the group dosed with M. avium, this organism was recovered in large numbers from the intestinal mucosa and mesenteric and the other lymph nodes of all calves. The avian tubercle bacillus was also isolated from the mesenteric nodes of the DTH-positive control calf.

## 4.2 Pathogeneses of paratuberculosis and avian tuberculosis in sheep

Fifteen 10-day old Scottish Blackface lambs obtained from the Institute's flocks were used for the investigation. Six animals were dosed with M. ptbc and six with M. avium. The remaining three were used as uninfected control animals, and were given sterile Reid's liquid medium. The same isolates of mycobacteria and protocol of infection used for the deer experiment (section 4.1) were employed and the necessary precautions were taken to prevent cross-infection between the groups. The animals were subjected to the various examinations described in section 4.1, following the same testing methods. Except for DTH, which was tested during weeks 6 and 14, all the other examinations were carried out following the same regimen. All animals were killed during week 19. Table 4.5 summarizes the results of the various examinations.

### 4.2.1 Clinical signs

In all groups, there were no obvious specific clinical abnormalities other than an unkempt wool coat in some of the infected lambs.

### 4.2.2 Faecal excretion of organisms

Organisms were found in ZN-stained faecal smears in some of the lambs dosed with M. ptbc during weeks 9-13, and only during week 9, in some of the lambs dosed with M. avium (Table 4.6). No organisms were detected in faeces in the control group.

4.2.3 Delayed-type hypersensitivity

All lambs dosed with M. avium reacted to the skin test during weeks 6 and 14. Those dosed with M. ptbc were only positive during week 14 (Table 4.7). Control lambs were negative on both occasions. As determined by the increase in skin fold thickness, the reaction to avian tuberculin PPD in both infected groups was much higher than that induced by the bovine PPD. However one of the lambs (No. 1771) dosed with M. avium had nearly equal reactions to both PPDs.

4.2.4 Serology

In both infected groups and the control lambs, there was no significant antibody response to the organisms (Table 4.5).

4.2.5 Pathology

4.2.5.1 Lambs dosed with M. paratuberculosis

(1) Gross pathology

In all lambs, there were no obvious external post-mortem abnormalities. Specific macroscopic lesions were found only in the intestines and mesenteric lymph nodes (Table 4.8). In comparison, lesions in the intestines were scanty and less obvious than those of the nodes, and were limited mainly to the small intestine.

(i) Intestines

Gross intestinal lesions occurred in all lambs. Significant lesions included enlargement of Peyer's patches and slight, diffuse thickening and congestion of the mucosa of the ileocaecal valve and terminal portion of the ileum. Occasional discrete nodules

measuring 2-3 mm in diameter were found in the jejunum in three of the lambs (Fig. 4.13).

(ii) Mesenteric lymph nodes

In all lambs, mesenteric lymph nodes were obviously enlarged, pale and oedematous. Some of them were indurated and they contained greyish-white foci which were gritty on cut-section.

(2) Histopathology

Microscopic lesions were mainly found in the intestines and mesenteric lymph nodes (Table 4.9). In only one lamb (No. 1793), were lesions found elsewhere, and that was in the retropharyngeal lymph nodes.

(i) Intestines

Lesions in the intestines were localized and were found in the ileocaecal valve, terminal ileum and the discrete nodules. In Peyer's patches, the lymphatic follicles were enlarged and hyperplastic (Fig. 4.14). Tuberculoid and extensive granulomas were present in the submucosa in the region of the Peyer's patches. The enlarged lymphatic follicles together with the granulomas produced considerable localized thickening of the submucosa. Some of the granulomas contained varying numbers of multiprocessed epithelioid cells, a few giant cells and some macrophages (Fig. 4.15). The epithelioid cells and giant cells were deeply eosinophilic. Many of the granulomas were walled-off from the

surrounding submucosal tissue by granulation tissue. Several granulomas, especially the extensive ones, produced mostly by coalescence of a number of smaller granulomas, were necrotic (Fig. 4.16). In some of them, there was mineralization of the necrotic masses. There were occasional foci of a few epithelioid cells and macrophages in the lamina propria of the mucosa overlying the Peyer's patches, especially in the villous tips. Less frequently, the foci contained one or more giant cells. The discrete nodules were frequently located in the submucosa in close association with the lymphatic follicles. They also contained granulomas similar to those found in the terminal ileum and ileocaecal valve. Lesions in the large intestines were found in the caecum in four lambs and they consisted mainly of enlarged and hyperplastic lymphatic nodules. Acid-fast organisms were not found in ZN-stained sections of the intestines.

(ii) Mesenteric lymph nodes

Granulomatous lesions similar to those found in the intestines were seen also in the mesenteric lymph nodes. Some of them were very extensive and replaced much of the normal lymphoid tissue. The extensive lesions were frequently associated with necrosis, mineralization and reactive fibrosis (Fig. 4.17). As in the intestines, AFB were not apparent in ZN-stained sections of the nodes.

(iii) Other organs

The lesions found in the retropharyngeal lymph nodes were

similar to those found in the mesenteric lymph nodes. However they were mild and scanty and acid-fast organisms were not seen in ZN-stained sections.

4.2.5.2 Lambs dosed with M. avium

(1) Gross pathology

The carcases were apparently normal. The distribution and character of the macroscopic lesions were basically similar to those in the lambs in the group dosed with M. ptbc. In five of the lambs, gross lesions were apparent in both the intestines and mesenteric lymph nodes, and in one, they were obvious only in the mesenteric nodes (Table 4.8).

(2) Histopathology

Likewise, the character and distribution of the histological lesions were similar to those found in the lambs dosed with M. ptbc. Microscopic lesions were found in the mesenteric nodes of all the lambs and small intestines of five (Table 4.9). In the other lamb (No. 1775), enlargement and hyperplasia of the Peyer's patches was not associated with the presence of granulomas. Lesions in the large intestines were also found in the caecum in two, and were similar to those seen in lambs in the group infected with M. ptbc. Lesions in organs other than the intestines and mesenteric nodes were found also in the retropharyngeal lymph nodes. They occurred in five lambs, and were similar to those in the lambs infected with M. ptbc.

AFB were not seen in ZN-stained sections of the intestines, mesenteric and retropharyngeal lymph nodes.

4.2.5.3 Control lambs

The specific gross and histopathological lesions in the lambs in the two infected groups were not seen in the control lambs.

4.2.6 Culture of tissues

Organisms consistent with those used in the inocula were recovered only from two infected lambs, one from each group, in very few numbers. M. ptbc was recovered only from the intestinal mucosa of lamb No. 1787, and M. avium, from the intestinal mucosa and mesenteric lymph nodes of lamb No. 1791.

## DISCUSSION

The experimental infection of very young red deer calves and lambs with M. ptbc and M. avium produced clinical disease earlier (week 6-8 vs week 15) and in a higher proportion of calves (3/3 vs 3/5) infected with M. avium than those exposed to M. ptbc (Table 4.1). Although clinical signs were similar, the clinical course was shorter and fatalities higher (3/3 vs 2/5) in calves infected with M. avium than those infected with M. ptbc. The lambs monitored for the 19 weeks did not show obvious clinical abnormalities. The results suggest a higher susceptibility of very young deer to infection with M. ptbc and M. avium than very young sheep. In comparison with the findings in the lambs and in previous investigations in cattle (Gilmour et al., 1965) and sheep

(Brotherston et al., 1961; Kluge et al., 1968; Karpinski and Zorawski, 1975) experimentally infected with M. ptbc, disease signs were manifested in deer much earlier than in sheep and cattle. In short, infection with M. ptbc progresses more rapidly in deer than in sheep and cattle.

The earlier occurrence of clinical signs, shorter clinical course and higher fatalities observed in the group of deer infected with M. avium suggest that in very young deer, avian tuberculosis is more acute and severe than paratuberculosis. Previous publications on avian tuberculosis in deer have reported conflicting views on their susceptibility to the avian tubercle bacillus. While Blaxter and others (1974) reported high susceptibility of housed red deer to infection with the bacillus, the findings of Orr and co-workers (1978) indicated a low susceptibility of the species to infection with the organism. Orr and co-workers considered the action of undetermined predisposing factors in precipitating diseases or difference in the pathogenicity of different strains to be a possible explanation for the contrasting findings. It may also be worthy of note that in their study, Orr and others used yearlings and a single dose whereas in the experiments described in this chapter, very young calves and 10 weekly oral doses were used. In other ruminants, age-dependent susceptibility and resistance to infection with M. ptbc is known to occur (Hagan, 1938; Doyle, 1953; Rankin, 1959, 1961b, 1962; Larsen et al., 1975), and prolonged exposure is considered to be the likely event under field conditions (Brotherston et al., 1961; Gilmour et al., 1965). The older age

and inadequate exposure to infection are worthy of consideration among factors likely to have contributed to the lower susceptibility to M. avium of the deer used in their study.

Faecal excretion of organisms was apparent in deer infected with either of the organisms only during the period when clinical signs were apparent. However organisms were not detected in deer No. 166 infected with M. ptbc during the relapse period. In the lambs, faecal excretion of organisms was evident in both infected groups only during the dosing period (Table 4.6). Thereafter, organisms were detected only in lambs infected with M. ptbc, up to week 13. The close association between the occurrence of clinical signs and faecal excretion of organisms is in accordance with the findings of Gilmour and others (1978). However clinically normal paratuberculous animals have also been reported to shed the organisms (Larsen, 1973). Nevertheless, that does not appear to have happened in deer in this experiment, unless the organisms were too few to be detected by microscopy and in culture.

In the immunological examinations, DTH response was detected in calves dosed with M. ptbc during week 8 (Table 4.2), and in the lambs, it was detected earlier (week 6) in those dosed with M. avium than with M. ptbc (Table 4.7). In the subsequent skin tests, a weakening of skin hypersensitivity was observed in the group of deer infected with M. ptbc whereas in sheep, skin hypersensitivity in the second test remained strong in the group of lambs infected with M. avium and became equally strong in that infected with M. ptbc. It is unfortunate that calves in the group infected with M. avium died and could not be subjected to the skin test.

Antibodies to the organisms were detected in deer only in the group dosed with M. ptbc, and they began to rise during week 12 (Fig. 4.3). In the lambs, up to week 19 when killed, there was no significant antibody response in either of the infected groups. In the group of deer dosed with M. ptbc, CMI response as determined by skin hypersensitivity occurred earlier than antibody response. The findings are in accordance with those described previously in sheep and cattle infected with M. ptbc (Larsen, 1973; Bendixen, 1978). The CMI response is considered to correspond to the preclinical stage of infection and the humoral response to the clinical phase, and there is frequently an inverse relationship between the two immune responses. The relationship may explain the relatively early, high seropositivity and negative skin hypersensitivity of deer No. 107, and the reverse for deer Nos. 99 and 109, all dosed with M. ptbc (Table 4.2 and Fig 4.3). Unfortunately the relationship between the two immune responses could not be examined in the group of deer infected with M. avium. However up to week 8, they were seronegative. As reported in the literature (Bendixen, 1978; Chaparas, 1982), immunity to M. ptbc and other mycobacteria is considered to be predominantly cell mediated, and resistance to infection with M. tuberculosis and M. ptbc was correlated with the onset of hypersensitivity (Gray, Graham-Smith and Noble, 1960; Gilmour and Brotherston, 1966). These hypotheses could explain why all challenged lambs, with their strong skin hypersensitivity, remained clinically normal. The same may have applied to the two calves (Nos. 99 and 109) dosed with M. ptbc, which had an initial strong skin hypersensitivity.

The typical gross and histopathological lesions induced by M. ptbc and M. avium in deer were basically similar in distribution, being found mainly in the intestines and mesenteric lymph nodes. Lesions in the mesenteric nodes were apparent in all animals infected with either organisms. However they were most severe in the calves infected with M. avium. Intestinal lesions were present in all lambs dosed with M. ptbc and in 5/6 of those dosed with M. avium, but they were localized and frequently walled-off. In those lesions in lambs, organisms were inapparent or too few to be detected in the ZN-stained sections. The changes in the lambs were indicative of organization of the lesions, suggesting regression of infection. The same animals manifested a strong skin hypersensitivity and remained clinically normal. Gilmour and co-workers (1978) found a significant correlation between the presence of strong skin hypersensitivity and the low numbers of M. ptbc in tissues of sheep at autopsy. In the deer, intestinal lesions were obvious only in those animals killed when clinical signs were apparent. In those killed during weeks 36 and 48, intestinal lesions were either mild and localized or inapparent. Intestinal lesions were commonly found in the distal small intestine in close association with the Peyer's patches. Gilmour and co-workers (1965) described the intestinal mucosa to be the primary site of infection and multiplication of M. ptbc before spreading to the regional lymph nodes. The M-cells of the ileal-dome epithelium have been shown experimentally to be the site of entry of the organism into the mucosa (Momotani, Whipple, Thiermann et al., 1988). Although lesions in the retropharyngeal

lymph nodes were common in the lambs infected with M. avium, and together with tonsillar lesions in severely affected calves infected with either organism, the role of the two lymphatic organs in the pathogeneses of the two infections was not elucidated. Nevertheless the progression of the intestinal lesions in deer observed in this investigation, especially in the group infected with M. avium, where early and more advanced lesions were captured, is consistent with the observations of Gilmour and others (1965). Early lesions were focal and they contained aggregates of a few epithelioid cells and macrophages. The lesions were frequently located in the interfollicular zones of the Peyer's patches immediately below the muscularis mucosa and in the villous tips of the overlying mucosa. In these early stages, organisms were more numerous in the mucosa, especially in the villous tips than in the submucosa, suggesting spread of organisms from the mucosal surface to the inner layers. Advanced lesions were more confluent and the cellular infiltration more extensive, implying spread by contiguity, as suggested previously by Nisbet and others (1962). In some areas of the submucosa, focal and extensive necrosis associated with infiltration by numerous granulocytic leucocytes predominantly eosinophilic, were common features found in the more severely affected intestines and mesenteric lymph nodes. Some of the foci had evidence of mineralization. Also in the severely affected intestines, deep ulcers and microgranulomas containing the granulocytic leucocytes and mononuclear cells were found in the mucosa, and organisms were seen in large numbers within epithelioid cells, macrophages and the necrotic masses. Giant

cells were seen rarely in the intestinal lesions in deer infected with either organism , but they were common in those in lambs in both infected groups. The degenerative changes found in severe and in chronic cases are thought to be a result of an immunological response related to a hypersensitivity-type reaction to the antigens of the mycobacteria, rather than direct action of the organisms on the tissues (Merkal et al., 1972).

Lesions in organs other than the intestines and mesenteric nodes were found only in the severely affected deer. They were secondary lesions, and were present in the liver, spleen, kidneys and lungs and some carcase nodes. Spread of lesions into such organs is considered to be by lymphatic and haematogenous routes (Hallman and Witter, 1933; Levi, 1948; Taylor, 1953; Kluge et al., 1968).

In deer, organisms were recovered from the mesenteric nodes of all challenged calves and from the intestines of only the severely affected animals. In the lambs, organisms were recovered only from two, one from each infected group, and were very few in numbers (Table 4.5). The low recovery rate of organisms in the lambs agrees well with the observations of Gilmour and others (1978), on the correlation between strong skin hypersensitivity and low numbers of organisms in tissues. The low recovery of organisms in the group of deer infected with M. ptbc and killed at the end of the experiment also suggests regression of the infection.

The way one of the control calves got infected with M. avium remains a mystery. However the possibility of cross-contamination though remote, cannot be ruled out. As this was unlikely to have

been more than a single challenge, it gives further evidence of the susceptibility of young deer to infection with M. avium.

In conclusion, the observations on the progression of infection with M. ptbc in red deer and lambs made in this investigation are in agreement with the description of Gilmour and others (1978), that after the phase of intestinal infection, some animals become clinical cases sooner e.g. deer Nos. 78, 107 and 166 or later e.g. deer No. 166, some remain infected for long periods without developing the disease e.g. deer Nos. 99 and 109, and others do recover. Complete recoveries were not encountered in deer in this investigation, however in animals which survived to the end of the experiment, organisms were inapparent or very few in numbers and were localized in the mesenteric nodes. The findings thus suggest that, the pathogenesis of paratuberculosis in deer is basically similar to that of the disease in other ruminants. However the disease in deer progressed more rapidly than in sheep.

Comparing infections with the two mycobacteria in very young deer, avian tuberculosis appeared to be more acute and severe than paratuberculosis. However the pathogenesis was basically similar.

The findings from the investigations on the pathogeneses of avian tuberculosis in red deer and sheep suggested that deer have high suseptibility for M. avium infection, and that the disease is more severe in very young deer than in very young sheep. However they also indicated that M. avium is a potential pathogen for sheep.

# CHAPTER 5

# Cultural Characteristics of Isolates of *Mycobacterium paratuberculosis* from Red Deer

## INTRODUCTION

The main characteristics of classical bovine isolates of *M. ptbc* which distinguish them from other mycobacteria with similar colonial morphology are their slow growth (8-12 weeks of incubation) and requirement for mycobactin during primary isolation on artificial media (Merkal and Curran, 1974). Several variants of *M. ptbc* identified in sheep (Taylor, 1951; Gunnarsson, 1979) and goats (Gunnarsson and Fodstad, 1979) differ from the classical bovine isolates in growth properties, growth rate, colonial morphology and pathogenicity.

Little is known about isolates of *M. ptbc* from deer and other wild ruminants and their relationship to isolates of the organism from cattle, sheep and goats. The work described in this Chapter was therefore aimed at characterizing isolates of *M. ptbc* from red deer by their cultural characteristics. In addition, the isolate of *M. ptbc* used in the experiments described in Chapter 4 was tested for pathogenicity for laboratory animals.

## RESULTS

### 5.1 Cultural characteristics

Fifty-four isolates of *M. ptbc* (Table 5.1) made at the Institute (Appendix 4), from naturally infected red deer were

investigated. All primary isolations were made from mesenteric lymph node homogenates. During their primary growth on artificial medium, the cultures were investigated for their growth rate, colonial morphology and requirement for mycobactin for a period of up to 20 weeks of incubation. They were also compared with two other isolates of M. ptbc (M928 and M961) from naturally infected red deer supplied by the VIC at Edinburgh, a bovine isolate of M. ptbc (C390) from the MAFF, CVL, Weybridge and a caprine isolate (M235) from the VIC in Aberystwyth. The procedures used for cultivation and testing for mycobactin requirement have been described under section 2.2.

5.1.1 Growth rate

Visible growth was detected usually after 4-6 weeks of incubation. However in some cultures, growth was noticeable by the fourth week (Table 5.2). Growth in the latter category of cultures was usually heavier. Colonies were distinct usually after 6-8 weeks of incubation and maximal growth was achieved after 8-12 weeks of incubation. One (M928) of the two other isolates of M. ptbc from deer grew more readily and in greater abundance than the others. Its colonies were visible from the third week after inoculation, and maximal growth was achieved within 4-6 weeks of incubation. The bovine and caprine isolates behaved like the other isolates from deer.

5.1.2 Colonial morphology

Young colonies (6-8 weeks old) were small, ranging from pin-point size to two mm in diameter. The larger colonies were found in cultures with fewer competing colonies. Most were greyish-white or dull-white but some were yellowish-white. All were opaque. They were slightly raised, round, with irregular margins and a rough, granular surface. Because they were firm and greasy, they tended to adhere to each other when contiguous.

Older colonies (>16 weeks old) were larger, measuring up to 3-5 mm in diameter, dull-white, more opaque and dry. They were usually flat with irregular margins, rough and granular. At the centre of some of the colonies were nipple-like projections, and others had wrinkled dome-shaped elevations with crater-like depressions.

Colonies of isolate M928 were yellowish-white, round, smooth and soft, but those of isolate M961 and the bovine and caprine isolates were similar to those of the majority of isolates of M. ptbc from deer.

5.1.3 Requirement for mycobactin

AFB were isolated from deer tissues only on the medium containing mycobactin. Isolate M961 and the bovine and caprine isolates were also recovered only from the medium containing mycobactin, but isolate M928 grew on the media with and without the growth factor.

## 5.2 Pathogenicity for laboratory animals

Isolate JD88/110 of M. ptbc isolated from a naturally infected, clinically affected adult red deer hind (No. D25; Chapter 3) used for the experiments on the pathogeneses of paratuberculosis in red deer and sheep (Chapter 4), was tested for pathogenicity for rabbits, guinea pigs and domestic fowl. The isolate was tested in parallel with the bovine isolate C390 and isolate M928 for comparative pathogenicity. The protocol for infection of the laboratory animals has been described under section 2.3.3. After challenge, the animals were monitored for clinical abnormalities for a period of nine weeks and then necropsied. Tissues were collected from the sites of injection and their related lymph nodes, along with intestines, mesenteric lymph nodes, spleen, liver and lungs, and processed for histopathology and culture following the procedures described in Chapter 2.

### 5.2.1 Clinical observations

None of the animals and fowls developed obvious clinical abnormalities within the 9-week observation period.

### 5.2.2 Pathology

Granulomatous lesions were found at the site of injection and the draining popliteal lymph nodes of the three guinea pigs injected with isolate M928. No granulomatous lesions were found in

the rabbits and fowls injected with the same isolate and in the other animals and fowls injected with the other deer isolate or the bovine isolate.

5.2.3 Culture of tissues

Mycobactin-independent AFB were recovered in moderate to large numbers from samples collected from the granulomatous tissues of the guinea pigs injected with isolate M928, and in small numbers, from the spleens of both fowls and one of the guinea pigs, the intestinal mucosa of one of the fowls and the liver of the other, injected with the same isolate (Table 5.3). No AFB were recovered from tissues collected from the animals and fowls injected with isolate JD88/110. In those given the bovine isolate, mycobactin-dependent AFB were recovered only from the spleen of one of the two rabbits.

## DISCUSSION

There were no obvious differences in cultural characteristics between the isolates of M. ptbc from deer or between the isolates from a cow and a goat. The organisms were slow-growing requiring up to 8-12 weeks of incubation for the colonies to achieve maximum growth, and were mycobactin-dependent. Their growth requirements and properties including colonial morphology were therefore consistent with those of the classical strains of M. ptbc from cattle (Merkal, 1970; Merkal and Curran, 1974). The findings are similar to those reported from New Zealand (Gumbrell,

1987), where culturally, strains of M. ptbc isolated from cases of paratuberculosis in deer resembled bovine strains. The earlier growth observed in some of the isolates (Table 5.2), which was also associated with heavier growth is more likely to have been due to culture from heavily infected tissues rather than to strain differences.

Isolate M928 which was not isolated initially at the Institute behaved atypically. It grew more readily than the other isolates in media with or without mycobactin and its colonies were smooth. However it is reported to have been slow-growing and mycobactin-dependent during primary isolation on artificial medium (Mathews, personal communication). Thus the isolate appears to have lost these properties of M. ptbc with subcultivation, a phenomenon reported previously by Merkal and Curran (1974) and Whipple and others (1987) for some isolates from cattle.

None of the three isolates of M. ptbc tested for pathogenicity for rabbits, guinea pigs and domestic fowl produced disease in them within the 9-week observational period. However isolate M928 produced granulomatous lesions at the sites of injection and in the draining lymph nodes in the guinea pigs, and moderate to large numbers of the organism were recovered in culture from those sites.

In conclusion, the isolates of M. ptbc from deer could not be distinguished from one another or from the isolates of the organism from a cow and a goat by cultural examination. However the variation in pathogenicity for laboratory animals and the difference in cultural characteristics shown by isolate M928,

suggest that this isolate may be different from the other isolates investigated in this study. Possible explanations are that it was either a variant of M. ptbc or an entirely different species of mycobacterium. A comparison of this isolate with the others at the molecular level may help to clarify the situation.

# CHAPTER 6

## Molecular Characterization of Isolates of *Mycobacterium paratuberculosis* from Red Deer

### INTRODUCTION

Various methods other than cultural characterization are in use for distinguishing mycobacteria and for comparing strains. A number of methods have been discussed in the review of the literature in Chapter 1, and the most promising appear to be in the field of molecular biology.

As determined by their growth rates, colonial morphologies and requirement for mycobactin, the 54 isolates of *M. ptbc* from deer described in Chapter 5 were shown to have cultural characteristics indistinguishable from those of isolates of the organism from a cow and a goat.

In the work described in this Chapter, attempts were made to characterize a selected number of the isolates by molecular biological methods. They were selected to include isolates from clinical and sub-clinical cases of paratuberculosis from Farms A and B. The isolates were characterized and compared with the isolates of *M. ptbc* from other ruminants, and other mycobacteria some of which were associated with a paratuberculosis-like disease syndrome in deer. The methods used were analysis of their cell proteins by SDS-PAGE and Western blotting, techniques used

previously by Bech-Nielsen, Burianek, Spangler, Heider, Hoffsis and Dorn (1985) and analysis of RE digestion and Southern hybridization patterns of their genomic DNAs.

## RESULTS

### 6.1 SDS-PAGE and Western blotting patterns of cell proteins of isolates of M. paratuberculosis from red deer

#### 6.1.1 SDS-PAGE patterns

Cell lysates of 14 isolates of M. ptbc from red deer (Appendix 4) selected from those described in Chapter 5 were analysed by SDS-PAGE and compared with isolates of M. ptbc from a cow (C390) and a goat (M235), the mycobacterial isolate M928 (atypical, as determined by cultural characterization and pathogenicity for laboratory animals), a commercial protoplasmic antigen (PPA-3; Allied Laboratories Inc. USA, P.O. Box 1063, Ames, IA) made from a bovine strain of M. ptbc (18M) and nine isolates of M. avium from red deer (4) and birds (5) (Appendix 5). The lysates were prepared, electrophoretically separated, and stained, following the procedures described in section 2.4.1.1.

Over 20 polypeptides, ranging in size from <20 kDa to 205 kDa, were observed in cell lysates of the isolates of M. ptbc from deer. The polypeptide profiles of the bovine and caprine isolates were similar to those of the deer isolates (Fig. 6.1). Similar polypeptide profiles were shown by the PPA-3 and the lysate of M928. However they differed from those of the M. ptbc lysates by exhibiting a doublet of approximately 56-58 kDa and by the absence

of some of the polypeptides in the 40 kDa to 45 kDa region (Fig. 6.2). The PPA-3 in addition, exhibited a polypeptide of approximately 38-40 kDa which was not present in the lysates of the M. ptbc isolates and M928 (Fig. 6.1 and 6.2). The M. avium lysates exhibited polypeptide profiles similar to those of M. ptbc (Fig. 6.1). However the major difference between the M. avium and the M. ptbc and M928 isolates was the presence of a polypeptide of approximately 38-40 kDa in the M. avium lysates which was similar to that shown by the PPA-3 (Fig. 6.3).

### 6.1.2 Western blotting patterns

The SDS-PAGE fractionated lysates of the mycobacterial isolates and PPA-3 described in section 6.1 were transblotted electrophoretically from the gels onto transfer membranes and probed in parallel with paratuberculosis-positive red deer (PPDS) and sheep (PPSS) sera, avian tuberculosis-positive (APDS) red deer serum, and red deer and sheep sera negative (negative control) for the two infections, following the procedures described in section 2.4.1.2. The positive sera were from naturally infected animals and their antibody titres were determined by ELISA.

#### 6.1.2.1 Blots probed with paratuberculosis-positive deer serum

Over 10 major polypeptides of molecular size within the range of <25 kDa to 205 kDa were recognized by the PPDS in lysates of the M. ptbc isolates from deer, the most prominent being those within the range of approximately 25 kDa and 66 kDa (Fig. 6.4). A similar

polypeptide pattern was exhibited by blots of the bovine and caprine isolates (Fig. 6.5). Isolate M928 exhibited a pattern of polypeptides similar to that of the M. ptbc isolates, but with fewer obvious polypeptides (Fig. 6.4 and 6.5). A polypeptide of approximately 116 kDa seen on blots of the M. ptbc lysates, occurred at a slightly higher level on that of M928 (Fig. 6.4).

The polypeptides recognized on blots of the PPA-3 and the M. avium isolates were also of similar pattern to those of the M. ptbc isolates and M928, but differed from them by the presence of the 38-40 kDa polypeptide which was not evident on blots of the isolates of M. ptbc and M928 (Fig. 6.5) and by the absence and weak expression of some of the polypeptides within the 40 kDa to 56 kDa range on blots of the PPA-3 and the M. avium isolates, which were obvious on those of the M. ptbc isolates. Two polypeptides in the regions of approximately 58 kDa to 66 kDa and 80 kDa to 97 kDa, were exhibited on blots of the PPA-3, but not on those of M928 and the M. ptbc and M. avium isolates (Fig. 6.5).

6.1.2.2 Blots probed with paratuberculosis-positive sheep serum

Over 12 major polypeptides within the range of <20 kDa to 205 kDa were recognized by the PPSS on blots of the M. ptbc isolates from deer, the most pronounced being those between approximately 25 kDa and 66 kDa (Fig. 6.6). The bovine and caprine isolates exhibited a polypeptide pattern similar to that of the deer isolates (data not shown). On the blot of M928, the serum also recognized polypeptides in a pattern similar pattern to that of the

M. ptbc isolates. However some of the polypeptides within the 29 kDa to 45 kDa and 66 kDa to 116 kDa ranges were of very low intensity, and some were inapparent e.g. the polypeptide of approximately 116 kDa (Fig. 6.6).

On blots of the M. avium isolates, the serum recognized polypeptides in a pattern similar to that of the M. ptbc isolates, but with polypeptides within the 20 kDa and 45 kDa region weakly expressed (data not shown). The PPA-3 was not analysed.

6.1.2.3 Blots probed with avian tuberculosis-positive deer serum

Polypeptides in a similar pattern were recognized by the APDS on blots of M928, the PPA-3 and the M. ptbc and M. avium isolates (Fig. 6.7 and 6.8). However as with the blots probed with the PPDS (section 6.2.1), the 38-40 kDa polypeptide was present only on the PPA-3 and M. avium blots and the two polypeptides within 58 kDa to 66 kDa and 80 kDa to 97 kDa were again present only on the PPA-3 blot (Fig. 6.7).

6.1.2.4 Blots probed with the negative control deer and sheep sera

(1) Negative control deer serum

Six polypeptides in a similar pattern within the range of 29 kDa to 205 kDa were recognized by the negative control deer serum on blots of the M. ptbc isolates from deer (data not shown). The polypeptides were also recognized by the PPDS at a higher intensity (Fig. 6.4). Similar polypeptide patterns were exhibited by the bovine and caprine isolates (data not shown). Polypeptides in a

pattern similar to that of the M. ptbc isolates was recognized by the negative control serum on the blot of M928. However some polypeptides on the blot of M928, were faint e.g. a 97 kDa polypeptide, and some while apparent on the M. ptbc blots e.g. two polypeptides of approximately 50 kDa and 80 kDa, were not evident. The polypeptides recognized on blots of the PPA-3 and M. avium isolates were also similar to those of the M. ptbc isolates (data not shown). However the presence of the polypeptide of approximately 38-40 kDa, exhibited only by the PPA-3 and the M. avium isolates, was the main difference between them.

(2) Negative control sheep serum

Four polypeptides of approximately 25 kDa, 29 kDa, 62 kDa and 205 kDa, in an identical pattern were recognized by the negative control sheep serum on blots of the M. ptbc isolates and M928 (data not shown). The polypeptides were also recognized by the PPSS at a higher intensity (Fig. 6.6). Similar polypeptides were recognized by the negative control serum and the PPSS on blots of the isolates of M. ptbc from a cow and a goat (data not shown). Only two (62 kDa and 205 kDa) of the four polypeptides were present on blots of the M. avium isolates (data not shown).

## 6.2 Restriction endonuclease digestion and Southern hybridization patterns of genomic DNA of isolates of M. paratuberculosis from red deer

Six isolates of M. ptbc from red deer analysed by SDS-PAGE

and Western blotting (Appendix 4) selected were also analysed for RE digestion and Southern hybridization patterns of their genomic DNAs. They were analysed along with the bovine (C390) and caprine (M235) isolates of M. ptbc, the mycobacterial isolate M928 and the nine isolates of M. avium (Appendix 5), for comparison. The organisms were grown and their genomic DNAs extracted, following the procedures described in section 2.4.2.1.

### 6.2.1 Genomic confirmation of M. paratuberculosis isolates

The isolates of M. ptbc were confirmed genomically by detection of the PCR-amplified unique 279 bp DNA fragment of the insertion element IS900, specific for M. ptbc. PCR amplification and assay of genomic DNA was performed on all the 18 mycobacterial isolates used in the study, following the procedures described in section 2.4.2.2.

Only the DNAs of the six isolates of M. ptbc from red deer and those of the bovine and caprine isolates displayed the specific fragment (Fig. 6.9), confirming that they were M. ptbc.

### 6.2.2 RE digestion patterns

Genomic DNA from all the 18 isolates of mycobacteria were restricted with the endonuclease BamHI (Gibco), but only those from the eight genomically confirmed isolates of M. ptbc (Fig. 6.9) were restricted with the enzymes PstI, HindIII and EcoRI (Gibco). Digestion of the DNAs with the restriction enzymes according to the supplier's specifications and separation of the restriction

fragments by gel electrophoresis were carried out following the procedures described in section 2.4.2.3.

A large number of fragments were observed for each of the enzymes BamHI, PstI and EcoRI and no differences were identified in the DNAs of M. ptbc made from all the eight isolates (data not shown). Repeated attempts to digest the DNAs with different stocks of HindIII were unsuccessful.

BamHI digests of DNAs of the M. avium isolates also produced a large number of fragments in identical patterns, but those of M928 were poorly resolved (data not shown). There were many similarities between the fragment patterns of the BamHI digested DNAs of the M. ptbc and M. avium isolates. However only on very close observation were differences noticeable.

### 6.2.3 Southern hybridization patterns

Only DNAs from the genomically confirmed isolates of M. ptbc (section 6.2.1) i.e. those made from deer (6), and of the bovine and caprine isolates, C390 and M235, respectively, were analysed. The BamHI, PstI and EcoRI DNA digests were transferred from the gels onto nylon membranes and hybridized to the $^{32}$P-PCR 279 probe, following the procedures described in section 2.4.2.4.

A characteristic hybridization pattern was observed for each of the enzymes used but no differences were identified between the deer isolates or the bovine and caprine isolates with the enzymes BamHI and PstI (Fig. 6.10, 6.11 and 6.12). The hybridization

pattern of the EcoRI digest of the bovine isolate was almost identical to that of the deer isolates and the caprine isolate, but differed from them by a RFLP of approximately 3.3 kb (Fig. 6.12).

## DISCUSSION

### SDS-PAGE and Western blotting

The protein profiles of the M. ptbc isolates from deer as determined by SDS-PAGE analysis were similar and indistinguishable from those of isolates of M. ptbc from a cow and a goat (Fig. 6.1), with the polypeptides being within the range of <20 kDa to 205 kDa. The polypeptide profiles of isolate M928 and the PPA-3 had many similarities to those of the M. ptbc isolates, but differed as well (Fig. 6.2). The M. avium isolates also had a polypeptide profile very similar to that of the M. ptbc isolates. However the major difference was the presence of a 38-40 kDa polypeptide in the M. avium isolates, shown also by the PPA-3, which was not present in the M. ptbc isolates (Fig. 6.1 and 6.3).

As determined by Western blotting, more than 10 of the SDS-PAGE separated polypeptides of the M. ptbc isolates from deer, cow and goat were recognized by PPDS and more than 12 by PPSS (Fig. 6.4 and 6.6). The polypeptide profiles for the various isolates of M. ptbc from deer were similar, with the most prominent polypeptides being those between <25 kDa and 66 kDa. However six of the polypeptides were recognized also by the negative control deer serum and four by the negative control sheep serum. The polypeptides observed between <25 kDa and 45 kDa on blots probed

with either the PPDS or the PPSS were nearly of equal intensity, but those between 45 kDa and 66 kDa were obvious only on blots probed with the PPSS. This suggests that the sheep is able to identify more antigenic polypeptides of mycobacteria than deer. Many of the polypeptides recognized by the PPDS and PPSS in isolate M928 were similar to those of the M. ptbc isolates. However fewer polypeptides were recognized in M928, with a polypeptide of approximately 116 kDa present in the M. ptbc isolates, either missing or displaced to a slightly higher molecular size level (Fig. 6.4 and 6.6). A polypeptide pattern similar to that of the M. ptbc isolates was produced on blots of the M. avium isolates probed with the APDS. However the main difference between them was the 38 - 40 kDa polypeptide which was recognized in the M. avium isolates and also the PPA-3, but not the M. ptbc isolates (Fig. 6.7 and 6.8). Polypeptides in a pattern similar to those of the M. ptbc and M. avium isolates and M928, were also recognized in the PPA-3 by the PPDS. However the PPA-3 differed by exhibiting two polypeptides within the region of approximately 58 kDa to 66 kDa and 80 kDa to 97 kDa (Fig. 6.5); these were not present in any of the mycobacterial isolates analysed.

Thus the findings from the SDS-PAGE and Western blotting analyses indicated a close similarity between the isolates of M. ptbc from deer and the isolates of the organism originating from a cow and a goat. They also showed that isolate M928 is an atypical form of M. ptbc or a species of a slow-growing mycobacterium other than M. ptbc. The 38-40 kDa polypeptide appeared to be a

consistent antigenic component of the M. avium isolates absent in the M. ptbc isolates. However the presence of the polypeptide in the PPA-3 raises a question as to the identity of the strain of mycobacterium (18M) from which the antigen was made. The findings on the PPA-3 are in accordance with those of Bech-Nielsen and others (1985), which showed the 38-40 kDa polypeptide in SDS-PAGE and Western blotting analyses of the PPA-3 but not of a sonicated bovine strain of M. ptbc (19698). Also from RE analysis of various strains of M. ptbc isolated from cattle, Collins and De Lisle (1986) found the strain from which the PPA-3 was made (18M) to differ from the others, and they considered it to be an atypical form of M. ptbc or a strain of another species of slow-growing mycobacterium. The recognition of more polypeptides by sheep immune serum suggests that the immune system of sheep is better able to identify more antigenic polypeptides of mycobacteria than the immune system of deer.

RE digestion and Southern hybridization

Each of the endonucleases BamHI, PstI and EcoRI produced a characteristic DNA fragment-pattern. The patterns of the isolates of M. ptbc from deer were identical to those of the isolates of the organism from a cow and a goat. The pattern of BamHI digests of the M. avium isolates was also very similar to that of the M. ptbc isolates. The DNAs resisted cleavage by HindIII. Failure of HindIII to digest mycobacterial DNA has been reported previously (Patel, Kvach and Mounts, 1986). Digestion by the enzyme was

resisted by DNA of a strain of M. tuberculosis (H4Ra), and modification by methylation at, or near, the HindIII recognition sequence was suggested as a possible cause for the failure.

Hybridization of the radiolabelled probe ($^{32}$P-PCR 279) to the DNA restriction fragments of the M. ptbc isolates from deer, produced characteristic patterns with each of the enzymes BamHI, PstI and EcoRI (Fig. 6.10, 6.11 and 6.12). The patterns were identical to those of the DNA of the goat isolate cleaved by the three enzymes, and to those of the bovine isolate DNA digested with BamHI and PstI. A RFLP of approximately 3.3 kb was seen in the hybridized EcoRI digests (Fig. 6.12), which differentiated the bovine isolate from the deer and goat isolates.

The observations from the RE analysis of the M. ptbc and M. avium isolates investigated support the close relationship of the two organisms reported previously by McFadden and others (1987). They found a 2% base substitution between M. ptbc and M. avium. The findings from the RE analysis and Southern hybridization of genomic DNAs of the selected isolates of M. ptbc from red deer indicate that the isolates analysed were homologous. They were also shown to be homologous to the isolate from a goat, but different from the isolate from a cow.

Assay of the isolates of mycobacteria used for the genomic study for the unique M. ptbc specific DNA fragment (PCR 279), detected the fragment only in the DNAs of the six isolates of M. ptbc from deer and those from a cow and a goat (Fig. 6.9). The absence of the fragment in the DNA of isolate M928 confirms that the isolate

is not M. ptbc. However the identity of this isolate remains to be ascertained. The unique fragment was also not exhibited by the M. avium isolates, thus further confirming the high specificity of the probe for the identity of M. ptbc reported by Moss and others (in press). The technique of RE analysis using a battery of enzymes in conjunction with hybridization with the probe may therefore find useful application in characterization of strains of M. ptbc in epidemiological studies of the organism.

# CHAPTER 7

## GENERAL DISCUSSION

In view of the paucity of knowledge of paratuberculosis of deer, the studies described in this thesis were designed to provide a better understanding of its aetiology, pathology, pathogenesis and epidemiology in red deer.

A high (38%) prevalence of infection of apparently normal, young (18 m.o. to 2 yr old) deer was found in an affected herd of red deer (Chapter 3). Experimental infection showed that the disease progressed more rapidly in deer than in sheep (Chapter 4), suggesting that M. ptbc is more pathogenic for deer than sheep. Similarly M. avium appears to be very pathogenic for deer. Thus the observations on red deer made from paratuberculosis and avian tuberculosis, when added to those in previous reports of the two infections and other mycobacterioses cited in this thesis, indicate that deer are susceptible to a wider range of mycobacteria than are cattle, sheep and goats. However most reports of paratuberculosis (Table 1.0) and other mycobacterial infections in deer and other wild ruminants (Rankin and McDiarmid, 1968) are from animals on farms, in parks and zoological gardens. Their susceptibility to mycobacterial infections under these intensive systems of management is enhanced by favourable conditions for the establishment and spread of such infections, such as high stocking rates and intensive rearing and housing. However as only limited information is available about paratuberculosis and other

mycobacterioses in free-living deer and other species of wild ruminants, the role they play in the epidemiology of such infections, is an area worthy of further investigation.

Poor bodily condition and emaciation were consistent clinical signs in deer affected by paratuberculosis (Chapters 3 and 4). This is in accordance with the manifestations of the disease reported previously for red deer (Bourgeois, 1940; Vance, 1961; Jørgensen and Jørgensen, 1987), other species of deer (Hillermark, 1966; Libke and Walton, 1975; Buxton, 1987) and other ruminants (Blood et al., 1983). However, although the pathological lesions varied, they were basically similar to those seen in other ruminants, especially sheep (Howarth, 1932; Stamp and Watt, 1954) and goats (Levi, 1948; Majeed, 1972). This was especially so in the severe cases, where the pathognomonic lesions in the intestines and related lymphatics, were associated with focal necrosis, mineralization and reactive fibrosis. Atypical lesions, which included haemorrhage in the intestinal mucosa and non-specific degenerative changes in the mesenteric lymph nodes, were the only pathological changes manifested by some of the severely affected animals. However as these animals were infected concurrently with intestinal nematodes and lung worms, the specificity of these lesions is unclear. Such cases of paratuberculosis are likely to be missed and diagnosed routinely as parasitic infections or other more obvious causes of ill-thriftiness unless the organism is cultured from the tissues or histological examinations are undertaken.

The clinical and pathological findings of avian tuberculosis in deer, as shown by a single natural case of the disease in an adult red deer hind (Chapter 3) and by experimentally infected calves (Chapter 4), were similar to those of paratuberculosis. However the lesions of avian tuberculosis were more severe and widely disseminated and not confined to the intestines and associated lymphatics. Similar severe lesions associated with avian tuberculosis in deer have been reported by Hopkinson and McDiarmid (1964). The close resemblance of the two infections is likely to complicate their diagnosis in deer. A nearly similar disease syndrome produced in deer in some cases of tuberculosis (Witte, 1940) and infection with the wood-pigeon mycobacterium (Jørgensen and Clausen, 1976), may cause even more confusion in the differential diagnosis of paratuberculosis in deer. Thus when dealing with suspected cases of paratuberculosis in deer, the other mycobacterioses are worthy of consideration, until the aetiology is confirmed.

By experimental infection of very young red deer and very young sheep with isolates of M. ptbc and M. avium made from naturally infected red deer (Chapter 4), the two infections were shown to progress more rapidly and more severely in deer than in sheep. Avian tuberculosis was more acute and severe than paratuberculosis in deer. The sheep dosed with either organism showed an earlier, stronger and more persistent skin hypersensitivity (Tables 4.2 and 4.7) than deer dosed with M. ptbc (deer challenged with M. avium were not skin tested). Also at necropsy, all

challenged animals had granulomatous lesions in their mesenteric lymph nodes in which AFB were demonstrated histologically and by culture, mostly from deer. Thus from the findings, it is suggested that the pathogeneses of paratuberculosis and avian tuberculosis in deer and sheep are basically similar, but the two infections progress more rapidly in deer than in sheep. And furthermore, very young deer react more severely than sheep to avian tuberculosis as well as to paratuberculosis. As immunity to mycobacterial infections is predominantly cell-mediated (Bendixen, 1978; Chaparas, 1982), the stronger CMI in sheep, indicated by the strong DTH determined by skin test, may explain the relative resistance of sheep to the two infections, when compared with deer. Further support to this hypothesis of a stronger immune response to M. ptbc of sheep than deer is provided by a higher ability to recognize more antigenic polypeptides of M. ptbc shown by the PPSS compared with the PPDS (Chapter 6). However the reasons for the relatively higher pathogenicity for young deer of M. avium compared with M. ptbc remain to be explored. One possible explanation could be that the relatively faster growing M. avium multiplies in tissues earlier than M. ptbc to reach large numbers sufficient to induce severe damage.

In an attempt to control paratuberculosis in an affected herd of deer (Chapter 3), vaccination of day-old calves with a standard bovine vaccine reduced significantly the rate (Table 3.1) and severity (Fig. 3.1) of infection, and the frequency of clinical cases of the disease in the herd. However the infection was not

eliminated from the herd within the three-year observational period. The findings are in accordance with previous observations on vaccination against paratuberculosis in other ruminants (Spears, 1959; Nisbet et al., 1962; Stuart, 1965a). However the long-term effects of vaccination on the infection in the herd remain to be elucidated.

The plethora of methods used to diagnose paratuberculosis is also worthy of comment. In the methods used for detection of infection in animals and tissues, faecal examination by microscopy and culture only detected infection in the clinically sick deer. The comparative i/d tuberculin test (using avian and bovine tuberculin PPDs) in deer, identified most (4/5) animals experimentally infected with M. ptbc (Table 4.2) before clinical signs were apparent. However the test failed to identify one severely affected animal and it did not detect all infected individuals when clinical signs were apparent and was negative thereafter. None of the seven clinically sick, naturally infected paratuberculous adult hinds investigated (Chapter 3) was positive with the skin test. While deer experimentally infected with M. avium were not tested, sheep experimentally dosed with the organism, showed an earlier DTH response than those exposed to M. ptbc. Serology by ELISA detected antibodies only in some of the M. ptbc experimentally (3/5) and naturally (2/7) infected, clinically sick deer. In sub-clinically infected animals the combination of histopathology and culture of mesenteric lymph nodes detected more cases of infection than either test alone (Table 3.2). The

findings thus support the views expressed in previous reports (Merkal, 1973; Thoen and Muscoplat, 1979; Chiodini et al., 1984a) on the diagnosis of paratuberculosis, that no single test is of sufficient sensitivity and specificity to detect all infected cases. The tests are of value mainly in detection of infection in the herd. However as the allergic tests (in vivo and in vitro) seem to be capable of detecting infection at an earlier pre-clinical stage than the others, they could be considered as the tests of choice for application to individual animals, for the detection of infection before it gets established in the animal and before the organisms are in turn excreted to spread to other susceptible individuals. A better understanding of CMI responses of deer induced by the various pathogenic mycobacteria of veterinary importance may help to improve, not only the immunodiagnosis of paratuberculosis and other important mycobacterial infections, but also their control by vaccination.

The cultural characteristics of isolates of M. ptbc from deer as determined by their growth rate, colonial morphology and requirement for mycobactin, were very similar, and were indistinguishable from those of a bovine and a caprine isolate of the organism (Chapter 5). In contrast, variants of the organism differing in cultural characteristics from the classical bovine isolates of M. ptbc, have been identified in sheep (Taylor, 1951 ; Gunnarsson, 1979) and goats (Gunnarsson and Fodstad, 1979).

A selected number of the isolates of M. ptbc from deer could also not be distinguished from one another or from the isolates

from a cow and a goat by SDS-PAGE and Western blotting (Chapter 6). However they were differentiated from an atypical isolate of mycobacterium (M928) from deer - atypical, as determined by its cultural characteristics and pathogenicity for laboratory animals, a commercial protoplasmic antigen (PPA-3) made from a bovine isolate of M. ptbc (18M) and isolates of M. avium. Despite the many similarities in the polypeptide-patterns of the isolates of the various mycobacteria analysed, the SDS-PAGE and Western blotting techniques were able to identify potential antigenic polypeptides for differentiation of the few species of mycobacteria investigated. However their usefulness in distinguishing strains of M. ptbc could not be ascertained in this study and therefore remains to be explored. Analysis of more isolates from different species and from a wider geographical distribution may provide a better assessment of their value.

On further characterization by RE digestion and Southern hybridization of the genomic DNAs (Chapter 6), of a few of the isolates of M. ptbc from deer previously analysed by SDS-PAGE and Western blotting, the isolates were shown with one (EcoRI) of the four (others were BamHI, PstI and HindIII) restriction enzymes used, to be homologous to a caprine but not to a bovine isolate of M. ptbc. This finding, when combined with the results from cultural examination and SDS-PAGE and Western blotting analyses, indicate that there are certain strain differences that are not expressed phenotypically, but can only be determined at the genomic level. The finding may be of significance for the epidemiological study described in Chapter 3 in that, prior to the

establishment of the deer herd on Farm A, there was an outbreak of paratuberculosis in a goat herd on the same farm. However the relationship of the isolates of M. ptbc from deer and the goat and other susceptible species has yet to be determined by a wider epidemiological investigation involving isolates of the organism from different animal species and from a wider geographical distribution.

In conclusion, the findings from the various investigations described in this thesis despite some variations, indicate that paratuberculosis of deer is basically similar to that of the disease in other ruminants, particularly sheep and goats. The high pathogenicity of M. ptbc, M. avium and other mycobacterioses for deer, indicates that mycobacterial infections are a threat to the deer industry and require serious attention and further research.

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APPENDIX 1. Buffers and solutions used

a. Phosphate buffered saline (PBS):

| | |
|---|---|
| Sodium chloride | 8.00g |
| Potassium chloride | 0.20g |
| Di-sodium hydrogen orthophosphate | 1.15g |
| Potassium di-hydrogen orthophosphate | 0.20g |
| Deionized DW to | 1000 ml |

b. ELISA coating buffer (ECB):

| | |
|---|---|
| Sodium carbonate | 1.59g |
| Sodium bicarbonate | 2.93g |
| Sodium azide | 0.20g |
| Deionized DW to | 1000 ml |
| Adjusted to pH 9.6 | |

c. Tris-borate-EDTA buffer (TBE, pH 8.3):

| | |
|---|---|
| Tris 7-9 | 10.80g |
| Boric acid | 5.50g |
| EDTA (di-sodium) | 0.93g |
| Deionized DW to | 1000ml |

d. Ficoll loading buffer (10X):

| | |
|---|---|
| 30% Ficoll 400 | 3.00g |
| 0.25% Bromophenol blue | 25mg |
| 0.25% Xylene cyanol FF | 25mg |
| 200 mM EDTA (di-sodium) | 0.74g |
| 10X TBE to | 10ml |

Heated to 100°C in a waterbath to dissolve and diluted to X5 prior to use.

e. Standard saline citrate (SSC; 20X):

| | |
|---|---|
| 3.0M Sodium chloride | 175.20g |
| 0.3M Tri-sodium citrate | 88.23g |
| Deionized DW to | 1000 ml |

Adjusted to pH 7.0 with citric acid or sodium hydroxide

f. Solutions required for radiolabelling DNA fragments:

(1) Oligo-labelling solutions

Solution A

| | |
|---|---|
| 10mM dCTP | 50 µl |
| 10mM dGTP | 50 µl |
| 10mM dTTP | 50 µl |
| 1M Magnesium chloride | 125 µl |
| 2M Tris pH 8.0 | 625 µl |
| Deionized DW | 82 µl |
| 2-mercaptoethanol | 18 µl |

Stored at -20°C

Solution B

2M HEPES titrated to pH 6.6 with sodium hydroxide

Stored at 4°C

Solution C

Hexadeoxyribonucleotides (Pharmacia P-L)

90 O.D. units/ml in 3mM Tris pH 7.0, 0.2mM EDTA

Stored at -20°C

(2) Oligo-labelling buffer

| | |
|---|---|
| Solution A | 20 µl |
| Solution B | 50 µl |
| Solution C | 30 µl |

Stored at -20°C

(3) Bovine serum albumin (BSA) 1.0 mg/ml
enzyme grade

(4) $\alpha$-dATP (Amersham 3000 Ci/mmol, 10 µCi/µl)

(5) Klenow (Pharmacia; 1u/µl)

(6) Stop solution

20mM Sodium chloride
20mM Tris HCl pH 7.5
2mM EDTA
0.25% SDS
1µM dATP

g. Pre-hybridization and hybridization buffer solutions:

Pre-hybridization buffer

3x SSC

5x Denhardt's solution

0.5% SDS

10µg/ml sheared denatured herring sperm DNA (HSDNA)

Made in a total volume of 20 ml

Hybridization buffer

Contained all the ingredients listed above except HSDNA.

Denhardt's solution (100x)

| | |
|---|---|
| 2% Ficoll 400 | 10g |
| 2% Polyvinylpyrrolidone (PVP) | 10g |
| 2% BSA (fraction V) | 10g |
| Deionized DW to | 500ml |

APPENDIX 2. Data for Fig. 3.1. The severity of infection with M. paratuberculosis in vaccinated and non-vaccinated red deer originating from an affected herd (Farm A) determined by mesenteric lymph node histopathology at slaughter

| Year | Vaccination Status | No. examined | No. positive | No. with severe lesions |
|---|---|---|---|---|
| 1st | NV | 167 | 64 | 41 |
| 2nd | V | 201 | 32 | 4 |
| | NV | 15 | 4 | 2 |
| 3rd | V | 47 | 13 | 1 |

V = vaccinated

NV = non-vaccinated

(Chi-square test: 41/64 vs 4/32 $p \ll 0.001$; 41/64 vs 1/13 $p \ll 0.001$; and 4/32 vs 2/4 $p = 0.24$).

APPENDIX 3. Data for Fig. 4.3. Summarized serological results (from ELISA) for red deer calves experimentally challenged with *M. paratuberculosis*

| Ear Tag No. | Optical densities (OD) during weeks | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | 4 | 8 | 12 | 15 | 19 | 22 | 26 | 31 | 36 |
| 78 | 0.031 | 0.023 | 0.034 | 0.031 | NT | 0.062 | 0.076 | 0.075 | 0.069 | 0.060 |
| 99 | 0.019 | 0.023 | 0.033 | 0.023 | NT | 0.020 | 0.009 | 0.021 | 0.016 | 0.032 |
| 107 | 0.007 | 0.009 | 0.021 | 0.093 | 0.133 | | | | | |
| 109 | 0.014 | 0.018 | 0.014 | 0.007 | NT | 0.013 | 0.012 | 0.009 | 0.035 | 0.010 |
| 166 | 0.008 | 0.007 | 0.011 | 0.035 | NT | 0.074 | 0.139 | 0.106 | 0.073 | 0.081 |

NT = not tested

Cut-off OD = 0.04

APPENDIX 4. Isolates of M. paratuberculosis from red deer made at the MRI

| Ref. No. | Source | Clinical/ Sub-clinical case | Tissue | Remarks |
|---|---|---|---|---|
| JD88/107 | Farm A | clinical | MLN & IM | a, b, c |
| JD88/110 | Farm A | clinical | MLN & IM | a, b, c |
| JD88/113 | Farm B | sub-clinical | MLN | a |
| JD88/114 | Farm B | sub-clinical | MLN | a |
| JD88/115 | Farm B | sub-clinical | MLN | a |
| JD88/116 | Farm B | sub-clinical | MLN | a |
| JD88/117 | Farm B | sub-clinical | MLN | a |
| JD88/133 | Farm B | clinical | MLN | a |
| JD88/134 | Farm B | clinical | MLN | a, b, c |
| JD88/135 | Farm B | clinical | MLN | a |
| JD88/163 | Farm A | sub-clinical | MLN | |
| JD88/175 | Farm B | clinical | MLN & IM | a, b, c |
| JD88/183 | Farm A | sub-clinical | MLN | |
| JD88/184 | Farm A | sub-clinical | MLN | |
| JD88/190 | Farm A | sub-clinical | MLN | |
| JD88/191 | Farm A | sub-clinical | MLN | |
| JD88/197 | Farm A | sub-clinical | MLN | |
| JD88/205 | Farm A | sub-clinical | MLN | |
| JD88/212 | Farm A | sub-clinical | MLN | |
| JD88/221 | Farm A | sub-clinical | MLN | |
| JD88/255 | Farm A | sub-clinical | MLN | |
| JD88/264 | Farm A | sub-clinical | MLN | |
| JD88/267 | Farm A | sub-clinical | MLN | |
| JD88/271 | Farm A | sub-clinical | MLN | |
| JD88/276 | Farm A | sub-clinical | MLN | |
| JD88/282 | Farm A | sub-clinical | MLN | |
| JD88/289 | Farm A | sub-clinical | MLN | |
| JD88/291 | Farm A | sub-clinical | MLN | |
| JD88/301 | Farm B | clinical | MLN & IM | a, b, c |
| Node Pool | Farm A | sub-clinical | MLN | a, b, c |
| JD89/5 | Farm A | sub-clinical | MLN | |
| JD89/11 | Farm A | sub-clinical | MLN | |
| JD89/46 | Farm A | sub-clinical | MLN | |
| JD89/49 | Farm A | sub-clinical | MLN | |
| JD89/53 | Farm A | sub-clinical | MLN | |
| JD89/68 | Farm C | sub-clinical | MLN | |
| JD89/69 | Farm C | sub-clinical | MLN | |
| JD89/71 | Farm C | sub-clinical | MLN | |
| JD89/82 | Farm A | sub-clinical | MLN | |
| JD89/106 | Farm A | sub-clinical | MLN | |
| JD89/110 | Farm A | sub-clinical | MLN | |
| JD89/116 | Farm D | sub-clinical | MLN | |
| JD89/124 | Farm A | sub-clinical | MLN | |
| JD89/129 | Farm A | sub-clinical | MLN | |

APPENDIX 4 (cont)

| Ref. No. | Source | Clinical/ Sub-clinical case | Tissue | Remarks |
|---|---|---|---|---|
| JD89/130 | Farm A | sub-clinical | MLN | |
| JD89/135 | Farm A | sub-clinical | MLN | |
| JD89/137 | Farm A | sub-clinical | MLN | |
| JD89/140 | Farm A | sub-clinical | MLN | |
| JD89/143 | Farm A | sub-clinical | MLN | |
| JD89/144 | Farm A | sub-clinical | MLN | |
| JD89/145 | Farm A | sub-clinical | MLN | |
| JD89/146 | Farm A | sub-clinical | MLN | |
| JD89/148 | Farm A | sub-clinical | MLN | |
| JD89/149 | Farm A | sub-clinical | MLN | |

a = analysed by SDS-PAGE and Western blotting
b = assayed by PCR
c = analysed by RE digestion and Southern hybridization
IM = intestinal mucosa
MLN = mesenteric lymph node

APPENDIX 5. Isolates of M. paratuberculosis and other mycobacteria from red deer and other species obtained mostly from other sources

| Ref. No. | Organism | Animal spp | Source | Remarks |
|---|---|---|---|---|
| C390 | M. ptbc | Bovine | CVL Weybridge | a, b, d |
| M235 | M. ptbc | Caprine | VIC Aberystwyth | a, b, d |
| M961 | M. ptbc | Cervine | VIC Edinburgh | a |
| M928 | M. ptbc | Cervine | VIC Edinburgh | a, b, c |
| AF1351/87 | M. avium | Cervine | CVL Weybridge | a, b, c |
| AF2362/87 | M. avium | Cervine | CVL Weybridge | a, b, c |
| AF3785/87 | M. avium | Cervine | CVL Weybridge | a, b, c |
| JD88/118 | M. avium | Cervine | MRI (Ex-Farm B) | a, b, c |
| AF416/88 | M. avium | Avian (chicken) | CVL Weybridge | a, b, c |
| AF1444/88 | M. avium | Avian (patridge) | CVL Weybridge | a, b, c |
| AF2341/88 | M. avium | Avian (chicken) | CVL Weybridge | a, b, c |
| AF667/89 | M. avium | Avian (white-cheeked turaco) | CVL Weybridge | a, b, c |
| AF668/89 | M. avium | Avian (garganey) | CVL Weybridge | a, b, c |

a = analysed by SDS-PAGE and Western blotting
b = assayed by PCR
c = analysed by RE digestion with only BamHI
d = analysed by RE digestion and Southern hybridization
CVL = Central Veterinary Laboratory
VIC = Veterinary Investigation Centre